OPTIMISATION OF IMAZALIL APPLICATION AND GREEN MOULD CONTROL IN SOUTH AFRICAN CITRUS PACKHOUSES

by

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Declaration

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Arno Erasmus Date

Dedication

This dissertation is dedicated to Ansa and the other part of us, Lukas, Klara and Anita “met al my liefde”.

Opsomming

Suid-Afrika is die grootste uitvoerder van verskeepde vars sitrusvrugte wêreldwyd. Een van die vernaamste faktore wat tot substansiële verliese kan lei, is na-oesverrotting. *Penicillium digitatum* (PD) en *P. italicum* (PI) is die hoof wondpatogene, en veroorsaak onderskeidelik groenskimmel- en blouskimmelverval. PD is meer algemeen as PI en daarom ook die fokus in die meerderheid van navorsing in hierdie veld.

Imazalil (IMZ) word deur die meerderheid van sitruspakhuise in 'n waterige doopbehandeling toegedien, en verskaf goeie genesende en beskermende beheer, sowel as sporulasie-inhibisie aktiwiteit. Twee IMZ-formulasies word gebruik: die sulfaatsout wat in waterige behandelings toegedien word, en die emulsifiseerbare konsentraat (EK) wat in wakslaagbehandelings toegedien word. Die meerderheid van navorsing op IMZ is gedoen deur die gebruik van die EK-formulasie. Die maksimum residu limiet (MRL) vir IMZ op sitrusvrugte is 5 $\mu g.g^{-1}$, terwyl 2-3 $\mu g.g^{-1}$ as 'n biologies effektiewe residuvlak beskou word wat ten minste groenskimmelsporulasie moet inhibeer.

'n Studie is uitgevoer ten einde die huidige status van IMZ-toediening in Suid-Afrikaanse pakhuise vas te stel, om die voldoende residuvlakke vas te stel wat nodig is om groenskimmel te beheer en sporulasie te inhibeer deur die gebruik van IMZ-sensitiewe en -weerstandbiedende isolate, en om optimisering van metodes van IMZ-toediening in sitruspakhuise te bestudeer. Faktore wat bestudeer is, was IMZ-konsentrasie, toedieningstipe (spuit vs. doop en stort), blootstellingsperiode, oplossingstemperatuur en pH, asook genesende en beskermende beheer van PD. Die pakhuis-opname het aangedui dat die meerderheid van pakhuise IMZ in 'n sulfaatsoutformulasie deur 'n fungisieddooptenk toegedien het, en 'n IMZ-residu van ≈1 $\mu g.g^{-1}$ gelaai het. In dooptoedienings het IMZ uitstekende genesende en beskermende aktiwiteit teen 'n *Penicillium* IMZ-sensitiewe isolaat gehad. Genesende beheer van 'n IMZ-weerstandbiedende isolaat was egter substansiëel minder, en beskermende beheer was verlore, selfs teen twee keer die aanbevole konsentrasie. Sporulasie is ook nie geïnhibeer nie. Die gebruik van natriumbikarbonaat (2%) gebufferde imazalil sulfaat-oplossings by pH ±8, in vergelyking met pH ±3 van die ongebufferde oplossings, het IMZ-residulading op nawel en Valencia lemoene merkbaar verhoog, en genesende en beskermende beheer van IMZ-weerstandbiedende isolaat verbeter. Blootstellingsperiode het nie IMZ-residulading in IMZ-sulfaat-oplossings by pH 3 geaffekteer nie, hoewel die MRL ná 45 s blootstelling in pH 8 oplossings oorskry is. Imazalil wat deur spuit- of drenkhandeling toegedien is, het residulading verbeter, maar groenskimmelbeheer was minder effektief as ná dooptoediening.

IMZ-formulasie (IMZ-sulfaat en EK), oplossing pH (IMZ-sulfaat teen 500 $\mu g.mL^{-1}$ gebuffer met $NaHCO_3$ of NaOH na pH 6 en 8) en blootstellingsperiode (15 tot 540 s) is daaropvolgend ondersoek ten einde IMZ-residulading en groenskimmelbeheer op Clementine mandaryn, suurlemoen, en nawel en Valencia lemoen vrugte te verbeter. Soos voorheen opgelet, het blootstellingsperiode geen betekenisvolle effek op residulading in die ongebufferde IMZ-sulfaat-oplossing (pH 3) gehad nie. Geen verskille is tussen die pH buffers wat gebruik is, waargeneem nie, maar residulading het met verhoogde pH verbeter. Die MRL is ná die doopbehandeling in IMZ EK (ná 75 s blootstellingsperiode), en IMZ-sulfaat by pH 8 en gebruik van $NaHCO_3$ (77 s) of NaOH (89 s) as buffer, oorskry. Die MRL is ná 161 s in IMZ-sulfaat-oplossings gebuffer by pH 6 met óf $NaHCO_3$ óf NaOH oorskry. Groenskimmelbeheer, soos beïnvloed deur residulading, is

gemodelleer ten einde beheer van IMZ-sensitiewe en IMZ-weerstandbiedende PD isolate te voorspel. Vanaf hierdie model is die effektiewe residuvlakke vir 95% beheer van 'n IMZ-sensitiewe isolaat en van 'n IMZ-weerstandbiedende isolaat as onderskeidelik 0.81 en 2.64 ug.g^{-1} voorspel.

Die effekte van inkubasieperiode (infeksie-ouderdom), blootstellingsperiode, oplossing pH, wondgrootte en borsel van vrugte ná doopbehandelings, op residulading en genesende groenskimmelbeheer, is ook ondersoek. Blootstellingsperiode het geen betekenisvolle effek op residulading op vrugte wat in pH 3 oplossings van IMZ (< 2.00 µg.g^{-1}) gedoop is, gehad nie. Verhoging van pH tot 6 het tot betekenisvolle verhoogde residulading gelei, wat met verlengde blootstellingsperiode toegeneem het, maar meestal tot vlakke onder die MRL ná 180 s. Ná-doop borsel van vrugte het residuvlakke wat in IMZ pH 3 oplossings verkry is, met tot 90% verminder na vlakke < 0.5 µg.g^{-1}; genesende beheer van die IMZ-sensitiewe isolaat was egter meestal ongeaffekteer, maar met swak sporulasie-inhibisie. By pH 6, het ná-doop borsel van vrugte residue tot ≈ 60% verminder; genesende beheer van die sensitiewe isolaat is weer nie geaffekteer nie, maar met verbeterde sporulasie-inhibisie. Gewonde skilsegmente het hoër residuvlakke gelaai in vergelyking met heel skilsegmente, en groot wonde het hoër vlakke gelaai in vergelyking met klein wonde (≈ 10.19, ≈ 9.06 en ≈ 7.91 µg.g^{-1} vir groot, klein en geen wond, onderskeidelik). Genesende beheer van infeksies wat vanaf groot wonde ontstaan het, was betekenisvol beter as dié vanaf klein wonde. Die vermoë van IMZ om sensitiewe groenskimmel-infeksies te beheer, het vanaf 6 en 12 h ná inokulasie op Clementine mandaryn vrugte van infeksies wat deur klein en groot wonde onderskeidelik geïnduseer is, afgeneem; op nawel lemoen vrugte, het genesende beheer 18 en 36 h ná inokulasie vir die onderskeie wondgrootte behandelings, afgeneem.

Effektiewe IMZ-konsentrasies wat 50% (EK_{50}) groei van nege PD en vyf PI isolate inhibeer, is *in vitro* vasgestel en die IMZ-sensitiwiteit van die verskillende isolate is volgens hul EK_{50} waardes en weerstandsfaktore (R) gekatogeriseer. Effektiewe residuvlakke wat 50% genesende ($ER_{50}C$) en beskermende ($ER_{50}P$) beheer van hierdie isolate voorspel, is *in vivo* vasgestel. Al die PI isolate het sensitiewe EK_{50} waardes van 0.005 - 0.050 µg.mL^{-1} gehad. Drie PD isolate was sensitief (0.027 – 0.038 µg.mL^{-1}), terwyl een weerstandbiedende isolaat as laag weerstandbiedend (R-faktor van 19) gekatogeriseer is, een as matig weerstandbiedend (R-faktor van 33.2), drie as weerstandbiedend (R-faktor van 50 - 57.6) en een as hoogs weerstandbiedend (R-faktor van 70.7). Sensitiewe PD isolate het gemiddelde $ER_{50}C$ en $ER_{50}P$ waardes op Valencia lemoen vrugte van 0.29 en 0.20 µg.g^{-1} gehad, en 0.33 en 0.32 µg.g^{-1} op nawel vrugte, onderskeidelik. ER_{50} waardes vir weerstandbiedende isolate het nie altyd met EK_{50} waardes gekorreleer nie en het van 1.22 – 4.56 µg.g^{-1} vir $ER_{50}C$ en 1.00 – 6.62 µg.g^{-1} vir $ER_{50}P$ waardes gevariëer. $ER_{50}P$ waardes vir weerstandbiedende isolate kon nie op nawel lemoen vrugte verkry word nie, maar $ER_{50}C$ waardes (1.42 – 1.65 µg.g^{-1}) was soortgelyk aan dié verkry op Valencia vrugte. Die PI isolate het almal soortgelyk aan die sensitiewe PD isolate opgetree, met $ER_{50}C$ en $ER_{50}P$ waardes op nawel en Valencia vrugte < 0.38 µg.g^{-1}. Alternatiewe swamdoders is vir die beheer van 'n IMZ-sensitiewe, -weerstandbiedende en -hoogs weerstandbiedende PD isolate getoets; hierdie het ingesluit: "sodium ortho-phenylpenate" (SOPP), thiabendazole (TBZ), guazatine (GZT), imazalil (IMZ), pyrimethanil (PYR) en Philabuster® (PLB; 'n kombinasie van IMZ en PYR), fludioxonil (FLU), azoxystrobin (AZO), Graduate®A^{+} ('n kombinasie van FLU en AZO) en propiconazole (PPZ). Veelvoudige weerstand is teen IMZ, GZT, TBZ en PPZ in beide weerstandbiedende PD isolate aangetoon. Vir die sensitiewe isolate, het IMZ, SOPP, TBZ, GZT en PLB die beste genesende beheer verskaf, terwyl IMZ, GZT en PLB die beste beskermende beheer verskaf het. Vir

die IMZ-weerstandbiedende isolate, het SOPP, PYR en PLB die beste genesende beheer verskaf, terwyl geen van die swamdoders voldoende beskermende beheer verskaf het nie.

Hierdie studie is wêreldwyd die eerste in-diepte studie van groenskimmel- en blouskimmelbeheer met die sulfaatformulasie van IMZ. Bevindinge vanuit hierdie studie word alreeds in die industrie geïmplementeer. Oplossing pH word gemonitor, blootstellingsperiode word gemeet en residulading spesifiek tot toedieningsmetode word bepaal en volgens die ER_{50} waardes geïnterpreteer. Waterige dooptoedienings het die beste ten opsigte van genesende beheer gevaar, en IMZ-residulading in wond-areas was die belangrikste vir genesende beheer. Ander studies het dit bevestig en getoon dat IMZ beter beskermend is wanneer in ‘n wakslaag toegedien word. Die praktiese impak van IMZ-weerstand is uitgelig aangesien weerstandbiedende isolaat-infeksies nooit voldoende beheer kon word nie. IMZ alternatiewe swamdoders is getoets en SOPP, TBZ, GZT, PYR en/of PLB kon gebruik word om die ontwikkeling en impak van IMZ-weerstand te verminder.

Summary

South Africa is the largest exporter of shipped fresh citrus fruit worldwide. One of the major factors that can lead to substantial losses is postharvest decay. *Penicillium digitatum* (PD) and *P. italicum* (PI) are the main wound pathogens, respectively causing green and blue mould decay. PD is more prevalent than PI and therefore also the focus in the majority of research in this field.

Imazalil (IMZ) is applied by the majority of citrus packhouses through an aqueous dip treatment, and provides good curative and protective control, as well as sporulation inhibition activity. Two IMZ formulations are in use: the sulphate salt applied in aqueous treatments and the emulsifiable concentrate (EC) applied with wax coatings. The majority of research on IMZ has been done using the EC formulation. The maximum residue limit (MRL) for IMZ on citrus fruit is 5 $\mu g.g^{-1}$, whereas 2-3 $\mu g.g^{-1}$ is regarded as a biologically effective residue level that should at least inhibit green mould sporulation.

A study was conducted to assess the current status of IMZ application in South African packhouses, to determine the adequate residue levels needed to control green mould and inhibit sporulation using IMZ sensitive and resistant isolates, and to study optimisation of modes of IMZ application in citrus packhouses. Factors studied were IMZ concentration, application type (spray *vs.* dip and drench), exposure time, solution temperature and pH, as well as curative and protective control of PD. The packhouse survey showed that the majority of packhouses applied IMZ in a sulphate salt formulation through a fungicide dip tank, and loaded an IMZ residue of ≈1 $\mu g.g^{-1}$. In dip applications, IMZ had excellent curative and protective activity against *Penicillium* isolates sensitive to IMZ. However, curative control of IMZ resistant isolates was substantially reduced and protective control was lost, even at twice the recommended concentration, nor was sporulation inhibited. The use of sodium bicarbonate (2%) buffered imazalil sulphate solutions at pH ±8, compared with pH ±3 of the unbuffered solutions, markedly increased IMZ residue loading on navel and Valencia oranges and improved curative and protective control of IMZ resistant isolates. Exposure time did not affect IMZ residue loading in IMZ sulphate solutions at pH 3, although the MRL was exceeded after 45 s exposure in pH 8 solutions. Imazalil applied through spray or drench application improved residue loading, but green mould control was less effective than after dip application.

IMZ formulation (IMZ sulphate and EC), solution pH (IMZ sulphate at 500 $\mu g.mL^{-1}$ buffered with $NaHCO_3$ or NaOH to pH 6 and 8) and exposure time (15 to 540 s) were subsequently investigated in order to improve IMZ residue loading and green mould control on Clementine mandarin, lemon, and navel and Valencia orange fruit. As seen previously, exposure time had no significant effect on residue loading in the unbuffered IMZ sulphate solution (pH 3). No differences were observed between the pH buffers used, but residue loading improved with increase in pH. The MRL was exceeded following dip treatment in IMZ EC (after 75 s exposure time), and IMZ sulphate at pH 8 using $NaHCO_3$ (77 s) or NaOH (89 s) as buffer. The MRL was exceeded after 161 s in IMZ sulphate solutions buffered at pH 6 with either $NaHCO_3$ or NaOH. Green mould control as influenced by residue data was modelled to predict control of IMZ-sensitive and IMZ-resistant PD isolates. From this model the effective residue levels for 95% control of an IMZ-sensitive isolate and of an IMZ-resistant isolate were predicted to be 0.81 and 2.64 ug g^{-1}, respectively.

The effects of incubation time (infection age), exposure time, solution pH, wounds size and fruit brushing after dip treatments on residue loading and curative green mould control were also investigated. Exposure time did not have a significant effect on residue loading on fruit dipped in pH 3 solutions of IMZ (<

2.00 $\mu g.g^{-1}$). Increasing the pH to 6 resulted in significantly increased residue loading, which increased with longer exposure time, but mostly to levels below the MRL after 180 s. Post-dip treatment brushing reduced residue levels obtained in IMZ pH 3 solutions by up to 90% to levels < 0.5 $\mu g.g^{-1}$; however, curative control of the IMZ sensitive isolate was mostly unaffected, but with poor sporulation inhibition. At pH 6, post-dip brushing reduced residues to ≈ 60%; again curative control of the sensitive isolate was unaffected, but with improved sporulation inhibition. Wounded rind sections loaded higher residue levels compared to intact rind sections and large wounds loaded higher levels than small wounds (≈ 10.19, ≈ 9.06 and ≈ 7.91 $\mu g.g^{-1}$ for large, small and no wound, respectively). Curative control of infections originating from large wounds was significantly better than those from small wounds. The ability of IMZ to control sensitive green mould infections declined from 6 and 12 h after inoculation on Clementine mandarin fruit of infections induced by small and large wounds, respectively; on navel orange fruit, curative control declined 18 and 36 h after inoculation for the respective wound size treatments.

Effective IMZ concentrations that inhibit 50% (EC_{50}) growth of nine PD and five PI isolates were determined *in vitro* and the IMZ sensitivity of the various isolates categorized according to their EC_{50} values and resistance (R) factors. Effective residue levels that predicted 50% curative ($ER_{50}C$) and protective ($ER_{50}P$) control of these isolates were determined *in vivo*. All the PI isolates had sensitive EC_{50} values of 0.005 - 0.050 $\mu g.mL^{-1}$. Three PD isolates were sensitive (0.027 – 0.038 $\mu g.mL^{-1}$), while one resistant isolate was categorized as low resistant (R-factor of 19), one as moderately resistant (R-factor of 33.2), three as resistant (R-factor of 50 - 57.6) and one as highly resistant (R-factor of 70.7). Sensitive PD isolates had mean $ER_{50}C$ and $ER_{50}P$ values on Valencia orange fruit of 0.29 and 0.20 $\mu g.g^{-1}$, and 0.33 and 0.32 $\mu g.g^{-1}$ on navel fruit, respectively. ER_{50} values for resistant isolates did not always correlate with EC_{50} values and ranged from 1.22 – 4.56 $\mu g.g^{-1}$ for $ER_{50}C$ and 1.00 – 6.62 $\mu g.g^{-1}$ for $ER_{50}P$ values. $ER_{50}P$ values for resistant isolates could not be obtained on navel orange fruit, but $ER_{50}C$ values (1.42 – 1.65 $\mu g.g^{-1}$) were similar to those obtained on Valencia fruit. The PI isolates all behaved similar to the sensitive PD isolates with $ER_{50}C$ and $ER_{50}P$ values on navel and Valencia fruit < 0.38 $\mu g.g^{-1}$. Alternative fungicides were assessed for the control of an IMZ sensitive, resistant and highly resistant PD isolates; these included sodium *ortho*-phenylpenate (SOPP), thiabendazole (TBZ), guazatine (GZT), imazalil (IMZ), pyrimethanil (PYR) and Philabuster® (PLB; a combination of IMZ and PYR), fludioxonil (FLU), azoxystrobin (AZO), Graduate®A^+ (a combination of FLU and AZO) and propiconazole (PPZ). Multiple resistance was shown against IMZ, GZT, TBZ and PPZ in both resistant PD isolates. For the sensitive isolates, IMZ, SOPP, TBZ, GZT and PLB provided best curative control, while IMZ, GZT and PLB provided best protective control. For the IMZ-resistant isolates, SOPP, PYR and PLB gave the best curative control, while none of the fungicides provided adequate protective control.

Globally, this is the first in-depth study of green and blue mould control with the sulphate formulation of IMZ. Findings from this study are already being implemented by industry. Solution pH is monitored, exposure time is measured and residue loading specific to application method is assessed and interpreted by means of the ER_{50} values. Aqueous dip applications performed best in terms of curative control, and IMZ residue loading in wound sites was most important for curative control. Other studies confirmed this and showed that IMZ is better protectively applied with wax coatings. The practical impact of IMZ resistance has been highlighted as resistant isolates infections could never be adequately controlled. IMZ alternative fungicides were assessed and SOPP, TBZ, GZT, PYR and/or PLB could be used to reduce the development and impact of IMZ resistance.

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CONTENTS

Chapter 1

AN INTRODUCTION TO THE SOUTH AFRICAN CITRUS EXPORT INDUSTRY, THE POSTHARVEST DISEASE GREEN MOULD AND THE FUNGICIDE IMAZALIL

1. The South African citrus export industry

From the first harvested fruit in 1661 by Jan van Riebeeck in the Company's garden in Cape Town and the first export consignment in 1906 (Cartwright, 1977), the South African citrus export industry grew to become the second largest exporter of fresh citrus fruit in the world after Spain (Edmonds, 2013). The 62238 ha under citrus production is divided into eight regions with distinctly different climate conditions. The four largest regions, each with more than > 8 000 ha under citrus production are located in the Limpopo, Western Cape, Mpumalanga and Eastern Cape provinces (Edmonds, 2013). The whole scope of citrus varieties is produced in South Africa. The bulk of the Valencia oranges and grapefruit are produced in the more subtropical areas, soft citrus and lemons are grown in the more Mediterranean and semi-arid climates, while navel oranges are produced in all three climate types (Edmonds, 2013). In 2012, South Africa exported more than 100 million cartons (15 kg) to more than 36 countries (Edmonds, 2013). As a result of the long distances to markets, the time for South African fruit to reach export markets can be from 6 (for soft citrus) to 12 weeks (for stronger varieties like oranges) (Pelser, 1977). Postharvest disease management is therefore of the utmost importance in order to assure the best possible quality fruit on the market. Green mould is one of the main contributors to postharvest losses in the South African export market due to decay (Pelser, 1977; Keith Lesar, personal communication).

In 1977, there was an estimated 150 packhouses in South Africa of which the majority (80) were smaller packhouses packing less than 50 000 cartons (15 kg) per season with 2 to 3 packhouses packing more than a million cartons per year (Pelser, 1977). In 2008 there were 316 packhouses of which 18 packed more than a million cartons and 35 between half a million and a million (Julius, 2009)

2. Green mould

2.1. Economical impact

Eckert and Eaks (1989) summarised research done on losses due to postharvest diseases where it was found that up to 82% of the total losses on a market could be ascribed to fungal diseases and that the worth of these losses due to decay could be millions of US dollars. They further stated that a criterion like "percentage disease" is not adequate in describing the "full economical impact" of these losses on the market to the producer. Shipments may have to be repacked or could be rejected because of a few diseased fruit; all these activities add to the cost of the specific shipment. Postharvest handling cost can be up to 70% of the total cost of a carton of export citrus fruit (Eckert, 1995). Green mould can be the cause for up to 90% of losses due to decay on the South African market [Christ, according to Eckert and Eaks (1989)].

2.2. Epidemiology

Penicillium digitatum needs a wound to successfully infect citrus fruit (Kavanagh and Wood, 1967); these wounds are usually induced during harvest or during the handling process in the packhouse (Rose et al., 1951; Brown, 1973). Wounds induced by insects in the orchard can also serve as entrances for the

fungus (Pelser, 1977). Inoculum in the form of conidia is produced in the orchard or packhouse from decaying fruit that was not removed during or after harvest time or that was discarded in the packhouse (Eckert, 1995). A single infection of *P. digitatum* can produce billions of conidia on one fruit (Holmes and Eckert, 1995). Conidia will mainly spread from these decaying fruit by means of air currents (Smilanick et al., 2006). Other sources of inoculum for the spread of diseases like green mould is the bins (containers) in which the fruit is transported from the orchard and stored in before packing, water used for cleaning, cooling, transporting and chemical treatment purposes, the air in and around the packhouse and the packline equipment (i.e. conveyor belts and brushes), which may have been in contact with decayed fruit (Eckert, 1977).

Fruit with fresh injuries induced during harvest or in the packhouse are the most susceptible for infection by *P. digitatum* (Eckert, 1995). Injuries induced in the flavedo of the citrus fruit peel in the absence of peel oil will produce lignin that prohibits infection from *P. digitatum* (Brown, 1973). Injuries deeper than the flavedo into the albedo are not able to produce lignin and will be more readily infected by *P. digitatum* (Brown, 1973). An injury, free water and nutrients are required for conidia of *P. digitatum* to germinate on citrus fruit (Green, 1932; Kavanagh and Wood, 1971; Pelser and Eckert, 1977). Fruit maturity and the virulence of the pathogen also play a role in the determination of whether green mould will develop or not in a specific wound (Eckert, 1995). Three days after infection and incubation at room temperature, a water soaked legion will be visible surrounding the infection site; after another 7 days, the whole rind of the fruit will be occupied by the fungus and green sporulation will be evident (Smilanick et al., 2006). *Penicillium digitatum* grows very slow below 10°C and will stop to develop at 1°C (Smilanick et al., 2006).

Conidia from green mould decaying fruit can contaminate neighbouring fruit in a packed carton situation, this term is called soilage and is undesirable from a marketing point of view (Smilanick et al., 2006). However, green mould will rarely spread from one fruit to another, except if the injury site of a decaying fruit comes into direct contact with a healthy fruit (Barmore and Brown, 1982; Smilanick et al., 2006). Galacturonic acid is exudated from the decaying wound and can induce a chemical wound if the rind of a healthy fruit has been slightly injured (by brushing or other packhouse treatments), thereby providing an entrance for green mould (Barmore and Brown, 1982).

During 1929 and 1930, an investigation was conducted in South Africa to find the reason for the excessive wastage due to decay that occurred on the South African export citrus. Mechanical injuries were singled out as the main reason. A list was made of all the possible areas where this injuries could be induced: “(1) Projections of various kinds and at various places in the machinery, e.g. dirt knobs, projecting screws and nails; (2) woodwork of machinery with sharp edges and splinters; (3) excessively long drops into the bins; (4) sizers badly neglected and hence causing abrasions; (5) sharp edges of conveyer belts; (6) sharp corners; (7) projecting wires in picking bags; (8) projecting nails and sharp corners in bins; (9) fields boxes of rough wood and sometimes with projecting nails; (10) pulled fruit; (11) fruit with excessively long stems; (12) spinning fruit due to defect in machinery; (13) excessive bulge; (14) broken lids; (15) nails driven into fruit during strapping; (16) careless lidding; (17) box nails dropped into fruit cases amongst the fruit; (18) unnecessary machinery” (Pole Evans, 1930). A positive correlation was found between the packhouses with the most of above mentioned areas for possible injury to the amount of wastage overseas (Hahne, 1930). Eckert (1995) confirmed this by finding that the incidence of decay through green mould is directly related to the number of injured fruit and the inoculum level.

2.3. Host pathogen interaction

Very early research already reported that a wound in the rind of a citrus fruit is needed for *P. digitatum* to infect and that deeper wounds are more susceptible for infection than shallower ones. However, very shallow wounds such as caused by the scraping of a fingernail can be sufficient for infection depending on what part of the rind has been injured (i.e. oil glands) (Green, 1932; Nadel-Schiffmann and Littauer, 1956). The peel is layered and divided into two main layers with the flavedo on the outside, which is the part that reflects the colour of the rind and the inner albedo layer, which is usually white or colourless (Soule and Grierson, 1986). The flavedo consists of the epicarp, hypodermis, the outer mesocarp and oil glands. A complex layer, the cuticle containing epicuticular waxes, covers the flavedo on the outside. The albedo consists mainly of parenchymatous cells that are arranged in a loose network with large intercellular air pockets. When a wound is induced on the rind of a citrus fruit, volatile compounds are released, mainly from the flavedo, which include limonene, α-pinene, β-myrcene, acetaldehyde, ethanol and carbon dioxide (Eckert and Ratnayake, 1994). If conidia from *P. digitatum* are present in the wound, it will be stimulated to germinate by these volatiles (Eckert and Ratnayake, 1994; Droby et al., 2008). Limonene has the strongest stimulative effect on germ tube elongation when compared to the other compounds (Droby et al., 2008). It has also been found that nonanal and citral compounds of citrus oil stimulate conidia to germinate (French et al., 1978). Conidia will germinate in wounds in the flavedo, but without nutrients it will not infect and cause a lesion (Kavanagh and Wood, 1971). Substances like glucose, fructose and sucrose have a great effect on conidia germination, but no effect on infection and colonisation, other substances like pectin have a negative effect on germination, but promote infection (Kavanagh and Wood, 1971). Pelser and Eckert (1977) found that it is a combination of constituents in orange juice that promotes conidium germination. Citrus peel extract enhanced the growth of *P. digitatum*, indicating that a combination of compounds found in citrus peel promotes growth of this fungus (Stange et al., 2002).

The substances that promote germination are not necessarily responsible for the promotion of infection, which means that if conidium germination is initiated, it will not necessarily lead to infection (Kavanagh and Wood, 1971). Germination will take place in the pH range of 3.5 – 8.0, but germ tube growth was enhanced most at pH 4.0 – 5.5 (Pelser and Eckert, 1977). *Penicillium digitatum* produces high amounts of citric and gluconic acid, which reduced the pH from 4.6 – 4.8 in healthy rind tissue to 3.0 – 3.2 in decayed tissue (Prusky et al., 2004). In another study, it was found that conidium germination of *P. digitatum* is almost 100% in pH 4 – 7, but was reduced to 10% at higher pH levels (pH 9) (Smilanick et al., 2005). Furthermore, it was found that conidium germination was not sensitive to carbon dioxide and conidia will germinate at just below 95% carbon dioxide (Brown, 1922). The ability for conidia of *P. digitatum* to germinate declined rapidly as temperature and relative humidity is increased; conidia stored at a relative humidity of 95% and 50°C will have a mortality of 99% after 4.9 h (Smilanick and Mansour, 2007).

Macarisin et al. (2007) explains from other researchers' work that a host plant will react to pathogen attack by quickly producing reactive oxygen species of which hydrogen peroxide (H_2O_2) is an example. During this oxidative burst, H_2O_2 is released in the host cells and can then be involved in oxidative cross-linking of cell wall proteins, or in lignification, hypersensitive cell death, or trigger defence related genes in neighbouring cells or lastly it can be toxic to the fungus when it comes in contact with H_2O_2. *P. digitatum* is able to inhibit the production of H_2O_2 in host cells by secreting citric acid and overcomes host resistance during infection (Macarisin et al., 2007). Catalase, an antioxidant enzyme, is also produced by *P. digitatum* and will remove H_2O_2, thereby increasing the pathogenicity of the fungus (Macarisin et al., 2007).

The albedo is usually first to be macerated and not the flavedo (Barmore and Brown, 1980), this could be due to the fact that the cells in the albedo are less compact than those of the flavedo (Soule and Grierson, 1986; Eckert and Eaks, 1989). If oil glands are damaged when a shallow wound (≥ 0.5 mm deep) is induced, infection of the flavedo will occur, but even then the fungus will be more inclined to invade the albedo before colonising the rest of the flavedo (Bates, 1936; Kavanagh and Wood, 1967). The albedo is described as "a fertile culture medium" for the infecting fungus (Soule and Grierson, 1986). A deep wound (> 2 mm) and water seems to be the prerequisite for conidia of *P. digitatum* to successfully infect citrus fruit (Kavanagh and Wood, 1967). Kavanagh and Wood (1971) explained that the presence of pectin in rind and fruit extract stimulate the fungus to produce enzymes that break down pectin. Pectin forms part of the middle lamellae, which are mainly broken down in the process of infection and disease development (Bush and Codner, 1968). Barmore and Brown (1979) associated galacturonic acid, pectinmethylesterase (PME) and exopolygalacturonase (exo-PG) with decay on citrus fruit caused by *P. digitatum*. PME (a natural component of fruit) and exo-PG (produced by the fungus) are pectolytic enzymes that can indirectly play a role in the maceration of fruit tissue by degrading the pectin components (Barmore and Brown, 1979). Extracts from the juice and rind of infected fruit had a macerating effect on sound fruit, although no pectic enzymes were found in juice from infected fruit and it appears that the pectic enzymes in infected juice and rind are deactivated. However, galacturonic acid, a product of exo-PG, could be detected (Cole and WOOD, 1970). Barmore and Brown (1979) divided fruit tissue maceration into three phases: firstly, the collapse of the cytoplasm, followed by the swelling of the cell walls and then cell separation takes place. They ascribed the collapse of the cytoplasm and the swelling of the cell walls to the accumulation of galacturonic acid. Due to the damage to the cell it releases PME, which enhances demethylation, pectic acid is produced during this process, which serves as a substrate for exo-PG. Exo-PG plays a role in the degradation of pectin, an important structural building block of cell walls. In combination with intercellular hyphal penetration, maceration is established.

Mature fruits of most citrus species are susceptible to green mould infection. It was also observed that ripe fruit are more susceptible for green mould infection than less mature fruit (Green, 1932). The albedo of less mature and disease resistant lemons with a dark green colour had a pH of 5.6 and it decreased in more mature and disease susceptible lemons with a yellow colour to a pH of 5.1 (Smilanick et al., 2005). D'Aguino et al. (2006) found that the blood orange cultivars 'Tarocco' and 'Sanguinello' were more susceptible to green mould infections (89.2 and 76.7% infection, respectively) than 'Valencia Late' oranges (60.1%). It was mentioned by Smilanick et al. (2008) that mandarins were more susceptible to infection than lemons and oranges. Fruit degreened at high relative humidities (90 – 96%) at 30°C were much less susceptible to green mould infection than fruit degreened at lower humidities and temperatures (Brown, 1973).

Sun *et al*. (2011) was first to report the full mitochondrial genome sequence of *P. digitatum*. The similarities in the mitochondrial genome showed that *Penicillium* and *Aspergillus* spp. are closely related. Despite this close relationship, *P. digitatum* has specific differences that make it host specific to citrus. These differences may be in the categories of secretion proteins and CAZyme-encoding genes (Marcet-Houben *et al.,* 2012). Gonzalez-Candelas *et al*. (2010) studied the host reaction in terms of gene expression. The expression of genes involved in production of alkaloids such as caffeine, phenylpropnoids from which lignin can be derived, and isoprenes are increased in reaction to the pathogen

infection. Ballester *et al.* (2011) stated that the production of phenylpropanoids (flavonoids, coumarins and lignin) and ethylene may be part of host response that induces resistance to the pathogen.

2.4. Management

Eckert and Eaks (1995) describes the most effective and practical ways to control decay (of which green mould is part) as follows: reduce injury during harvest and packing; reduce *Penicillium* inoculum in the orchard and packhouse; apply broad spectrum fungicides such as chlorine or quaternary ammonium during the washing of the fruit and treat the fruit with a selective fungicide such as imazalil (IMZ) or thiabendazole (TBZ). Depending on the specific fungicide, the delay between harvest and fungicide treatment should be as short as possible as the risk of green mould development increase with time after harvest. Fruit temperature plays an important role in combination with fungicide application time. It has been found that fruit can be stored at 10ºC for up to 70 h or at 25ºC for up to 30 h before commencing IMZ treatment and green mould control should still be adequate (Wild and Spohr, 1989).

Another important aspect of disease management is the management of fungicide resistance by putting more emphasis on the use of alternative management options such as heat, sodium bicarbonate and chlorine, alternate organic fungicides with different modes of action, sanitation and monitoring of the resistance level in the targeted fungal population (Eckert, 1995). Eckert (1990) suggest 3 strategies to control wound pathogens: 1) disinfest the fruit and its environment; 2) control of germinating spores in wounds; 3) load a residue of fungicide in the wounds and healthy surface of fruit in order to prevent infection at a later stage. The fungicide that is used to a large extent against *P. digitatum* in citrus packhouses is IMZ.

3. Imazalil

3.1. History and mode of action

Imazalil was first tested for the control of green and blue mould in the late 1970's, when resistance to thiabendazole (TBZ) and benomyl became a major problem (Harding, 1976; McCornack and Brown, 1977). It became clear that IMZ is a very effective fungicide for the control of *Penicillium* green and blue moulds on citrus (Laville et al., 1977). Its ability to effectively control TBZ and benomyl resistant strains became one of its major advantages (Harding, 1976; Bus et al., 1991). In the light of this attribute, an almost 50% reduction in losses due to decay on export fruit from South Africa was attributed to the introduction of IMZ into the South African citrus industry (Pelser and La Grange, 1981). Imazalil also has some controlling effect against alternaria rot, stem-end-rot and sour rot (Laville et al., 1977; McCornack and Brown, 1977).

Imazalil (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy-ethyl]-1*H*-imidazole) is a demethylation inhibitor of ergosterol biosynthesis (Siegel and Ragsdale, 1978; Siegel, 1981). Sterols are essential for growth and reproduction of fungi and other organisms (Siegel, 1981). This group of fungicides do not have an inhibiting effect on the initial development of fungi, which includes spore germination, initial cell growth and dry weight increase, but rather causes irregularities in the growth patterns of fungi by means of changing the cell morphology through inhibition of ergosterol biosynthesis (Siegel, 1981). Despite the sensitivity of ergosterol biosynthesis to these inhibitors, normal development of the fungi will only be slightly retarded initially after treatment. This is because the biosynthesis of ergostorol is higher than the utilisation of it during membrane synthesis, but as soon as the ergosterol becomes depleted, normal growth is distorted or inhibited (Siegel, 1981). The majority of ergosterol biosynthesis inhibitors including IMZ, restrict the sterol C-14α

demethylation in the ergosterol biosynthesis pathway (Siegel, 1981).

A very important attribute of IMZ is its ability to inhibit sporulation (McCornack and Brown, 1977). This can prevent soilage and still render the neighbouring fruit of a decayed fruit marketable. After treatment, the majority (91 – 94%) of the applied IMZ is systemically absorbed but will remain in the exocarp (peel) (Brown et al., 1983). It was also found that degreened fruit do not absorb more IMZ than non-degreened fruit (Brown et al., 1983). IMZ applied in an aqueous solution has a better curing effect than protecting (Dore et al., 2009), less decay were evident when treatment happened 24 h after inoculation compared to 24 h before inoculation. The working of IMZ becomes more effective as the range of pH moves from 5.1 to 5.9 (Holmes and Eckert, 1999) and the effectiveness of IMZ to control conidium germination increase at higher pH levels (Smilanick et al., 2005).

IMZ is available in different formulations: emulsifiable concentrate (EC), imazalil nitrate salt and imazalil sulphate salt. These different formulations can be used at different concentrations for different application methods, for instance dipping, spraying or with the wax application (Laville et al., 1977). Although, few cases of phytotoxicity were reported, Schirra et al. (1997) found that IMZ applied at high concentrations (1000 $\mu g.mL^{-1}$) resulted in external damage on lemon fruit.

3.2. Application.

Imazalil can be applied by means of a dip, drench or spray in water or in a water-based wax solution (Laville et al., 1977; McCornack et al., 1977; Kaplan and Dave, 1979; Njombolwana et al., 2013a; Njombolwana et al., 2013b). In South Africa, a dip treatment for 1 to 3 min in 250 – 500 $\mu g.ml^{-1}$ IMZ was recommended as the most effective application method (Pelser and La Grange, 1981). Imazalil sulphate was recommended as the preferred formulation due to its water-solubility (Pelser and La Grange, 1981). Immersion of fruit in an aqueous solution of the IMZ EC formulation was significantly more effective than through wax application (Smilanick et al., 1997). Dip treatments in 500 $\mu g.ml^{-1}$ IMZ at 60 s gave similar decay control compared to a drench treatment at the same concentrations and application time, but was more effective in controlling sporulation (Kaplan and Dave, 1979). In other studies, spray application tended to be less effective when compared to drench and dip applications (Kaplan and Dave, 1979; Brown and Dezman, 1990). These authors also found that dip treatment in an aqueous solution gave the best results in terms of green mould control.

Smilanick et al. (1997) found that IMZ solution temperature, concentration and exposure time all have an effect on the subsequent loading of IMZ residues on fruit. Warmer temperatures, higher concentrations and longer exposure times will increase the IMZ residue on treated fruit. By using the EC formulation of IMZ, they found the ideal combination to be a solution temperature of 37.8ºC, at a concentration of 350 to 400 $\mu g.ml^{-1}$ and an exposure time of 30 s. An IMZ residue of 2 to 4 $\mu g.g^{-1}$ on fruit can be expected. A second alternative was suggested where the fruit could be exposed to the solution for 15 s at the same temperature and concentration with an additional wax treatment that contains IMZ at a concentration of 1000 $\mu g.ml^{-1}$ (Smilanick et al., 1997). Njombowana et al. (2013a) confirmed this showing that IMZ applied with the wax was better protective than curative and gave excellent inhibition of sporulation. Schirra et al. (1997) found that dipping lemon fruit in a much lower IMZ concentration, *i.e.* 50 µg.g-1, but at longer exposure (3 min) and higher solution temperature (50ºC) will give significant protection against citrus green mould.

Imazalil application by means of a fungicide dip tank can be troublesome in terms of maintaining the

desired concentration, fruit are constantly taking IMZ out of the solution and if the bath is topped-up with clean water the IMZ concentration will be reduced (Altieri et al., 2005). A method of spectrophotometry using near-infrared and one using ultraviolet wavelengths has been developed to accurately measure the concentration of IMZ in commercial fungicide baths (Altieri et al., 2005). In South Africa the majority of packhouses follow a generic top-up protocol where 5g of IMZ is added per ton of fruit processed every few hours during the operation of a packhouse (Lesar and Hardman, 2004). The most reliable method to manage the concentration of a dip tank is by means of titration. Through this method the exact concentration can be determined and the concentration can be readjusted to the registered concentration of 500 $\mu g.g^{-1}$ (Lesar, 2008). IMZ applied in an aqueous wax gave very good sporulation control but relatively poor decay control (Kaplan and Dave, 1979). In a later study, atomisers were used to apply IMZ in water and in water based wax solutions over rotating brushes and adequate residue levels were obtained; however, IMZ applied in water gave better results in terms of residue loading and green mould control (Brown et al., 1983; Brown, 1984). The more optimal application of IMZ in water will also give more protection against infections occurring after treatment (Brown, 1984).

IMZ in combination with sodium bicarbonate was very effective in controlling green mould caused by a sensitive and a resistant isolate of *P. digitatum* (Smilanick et al., 2005). Smilanick et al. (2005) gave four possible reasons for the improved effect of IMZ and sodium bicarbonate combination on the control of green mould: 1) the higher pH will increase the toxicity of IMZ; 2) the higher pH can inhibit the development of *P. digitatum*; 3) there might be synergy between the ability of IMZ and sodium bicarbonate to alter the membrane of *P. digitatum*; 4) the toxicity of sodium bicarbonate against *P. digitatum* and the possibility of sodium bicarbonate to interfere with the process of infection by making the wound environment unsuitable for enzyme secretion and/or activity.

3.3. Residue loading.

When fruit is treated with imazalil a residue is deposited on the fruit. This is important for a lasting control effect against green mould during transport and cold storage. After prolonged storage (13 weeks at 9°C and 1 week at 21ºC) the IMZ residue was degraded to 40% of the original residue on the fruit (Schirra et al., 1997). However, (Dore et al., 2009) reported that more than 75% of residue loaded at treatment generally remained on the fruit after storage and shelve life.

Depending on the incubation criteria and the sporulation rating, researchers found that an IMZ residue of 1 – 2 $\mu g.g^{-1}$ (Kaplan and Dave, 1979; Brown and Dezman, 1990; Eckert et al., 1994) or 2 – 3.5 $\mu g.g^{-1}$ (Smilanick et al., 1997) is necessary to control sporulation of green mould.

The epicuticular wax layer on the fruit rind does not play a role in the amount of residue that will be loaded on a specific fruit, but prevent some of the IMZ (40%) from penetrating deeper into the rind (Brown and Dezman, 1990). Importantly, it is the part of the residue that stays on the epicuticular wax layer that plays a major role in the inhibition of sporulation (Brown and Dezman, 1990). IMZ was detected to a depth of >1 mm into the rind 30 – 45 min after a 15 s treatment in 1000 $\mu g.mL^{-1}$ IMZ, but less than 1% moved beyond the cuticle layers of the flavedo (Brown and Dezman, 1990). IMZ penetrates into the albedo when applied in a heated (50°C) solution, but more than 80% stay in the flavedo (Dore et al., 2009).

Increasing the solution temperature, exposure time or IMZ concentration had an elevating effect on the IMZ residue loaded on the fruit when dip application was done (Brown and Dezman, 1990; Smilanick et al., 1997; Cabras et al., 1999; D'Aquino et al., 2006). Imazalil residue was doubled when the pH of the

solution was increased from 3 and 5 to 7 and 9 (Smilanick et al., 2005). It was suggested that the solubility of IMZ into the fruit cuticle and wax is increased with higher pH levels and this is probably the reason why IMZ residue loading increased with higher pH levels (Smilanick et al., 2005).

Drench, dip and aqueous IMZ applications gave the best residue loading results compared to spray and wax application (Kaplan and Dave, 1979). Interestingly, residue loading could not be doubled when the concentration was increased five times (Kaplan and Dave, 1979). Laboratory dip treatments were more effective in terms of residue loading and control of IMZ resistant biotypes of *P. digitatum* when compared to conventional water or wax treatments on brushes in commercial packhouses (Eckert et al., 1994). In recent work it was shown that higher residue levels can be achieved when IMZ is applied with wax compared to dip application with IMZ sulphate (Njombolwana et al., 2013a; Njombolwana et al., 2013b).

Temperature of the aqueous IMZ solution plays an important role in residue loading. The loading of IMZ on fruit during ambient temperature treatments was correlated to fruit maturity due to cracks that develop in the wax surface as maturity progress; more cracks will take up more IMZ than non-cracked surfaces. However, the loading of IMZ on citrus fruit during heated treatments was not correlated to fruit maturity (Schirra et al., 1996; 2000). Heated (50ºC) treatments induce altering of the wax layer and cracks disappear, therewith trapping IMZ (Schirra et al., 1997). In heated treatments, residue loading was therefore directly correlated to the IMZ concentration. Fruit treated in 50°C for 2 min in water developed more fractures in the cuticular layer; these fractures will increase in size as storage progress. IMZ will accumulate in these fractures if it were in the solution and will thus protect these areas from infection (Dore et al., 2009). With the ageing process these fractures will increase in size during storage and expose parts of the rind that have not come into contact with the fungicide and so will become entry points for infection (Dore et al., 2009). This is not the case for colder (25°C) IMZ solutions (Dore et al., 2009).

When IMZ is applied in combination with TBZ, less IMZ gets absorbed by the cuticular wax, which will in turn cause increased degradation of IMZ over time (Cabras et al., 1999). IMZ residue that is not absorbed by the cuticular wax will degrade over time, but those that do gets absorbed will be more persistent (Cabras et al., 1999). Small wounds take up more IMZ than uninjured rind when applied in water rather than water based wax (Brown et al., 1983; Brown, 1984). Large wounds induced by sandpaper will load the same IMZ residue when applied through water or water based wax (Brown, 1984). It appears that wounds attract more IMZ than uninjured rind. Dore et al. (2010) confirmed this by showing that wounded albedo took up 6 fold more IMZ than intact rind.

3.4. Resistance.

The level of resistance to IMZ in a variety of fungi tested appeared to be low, although there was a high frequency to mutate to resistance (van Tuyl, 1977). The potential of a fungus to develop resistance to a certain systemic fungicide is more dependent on the level of resistance after one mutation than on the frequency of mutation (van Tuyl, 1977). In light thereof, IMZ can be regarded as a more safe fungicide to use in terms of potential development of resistance (van Tuyl, 1977). Moreover, the potential to develop resistance to a certain fungicide is not necessarily equated to practical field resistance (*i.e.* loss of control). The fungus needs to be able to build up a sub-population of resistant isolates in the field where the virulence and fitness of these resistant isolates will play an important role in the survival of such a sub-population (van Tuyl, 1977). It is postulated that IMZ interferes with the permeability of the cell membrane; resistance is then obtained by modifying the composition of the sterols in the membrane (van Tuyl, 1977).

IMZ resistance development is described as the over-expression of the gene P*d*CYP51, which will increase the amount of P405-dependant sterol 14α-demethylase, which will in its turn affect IMZ-resistance (Hamamoto et al., 2000; Ghosoph et al., 2007; Kiralj and Ferreira, 2008). Resistance to IMZ is polygenic and involves 21 genes on 8 loci and is linked with 6 groups; this means that it is theoretically more difficult for *Penicillium* to develop resistance due to all the different genes involved (Laville et al., 1977). So far three CYP51 genes that contributes to IMZ resistance have been characterised: IMZ-R1 (Hamamoto et al., 2000), IMZ-R2 (Ghosoph et al., 2007) and IMZ-R3 (Sun et al., 2013).

During the late 1970's, losses due to green mould decay were almost doubled in California and the reason was attributed to fungicide (*i.e.* TBZ) resistance of *P. digitatum*. This led to an emergency clearance for the use of IMZ by the California Department of Agriculture (Anonymous, 1980). In California, citrus fruit are treated and packed all year round and are also repacked after prolonged storage, with decaying fruit being culled before packing. This practice favours the selection and promotion of IMZ-resistant isolates (Holmes and Eckert, 1999). According to (Holmes and Eckert, 1995), Eckert gave the first report of IMZ resistant strains of *P. digitatum* in 1987. Infection caused by resistant isolates cannot be controlled or its sporulation inhibited with normal packhouse IMZ treatments (Eckert et al., 1994). IMZ-resistant isolates of *P. digitatum* are generally less fit on fruit not treated with IMZ when compared to IMZ-sensitive isolates (Dave et al., 1989; Holmes and Eckert, 1995; Kinay et al., 2007). It has to be stated that in both these studies a small proportion of the IMZ-resistant isolates tested did not decline when in competition with IMZ-sensitive isolates on nontreated fruit (Holmes and Eckert, 1995; Kinay et al., 2007). This loss of fitness is only evident when the IMZ-resistant isolate is in competition with the IMZ-sensitive isolate and is not well understood (Holmes and Eckert, 1995). If constant use of IMZ is continued, the IMZ-resistant strains might overcome their fitness penalty due to the selection pressure (Holmes and Eckert, 1995). IMZ resistance may develop in variable spatial and temporal patterns throughout a citrus packing season (Ghosoph et al., 2007). Thus, emphasis should be placed on alternation between fungicides that have different modes of action in a resistance management program even if the packhouse and orchard is isolated and managed well.

In South Africa, the first resistant strains of *Penicillium digitatum* against benomyl and thiabendazole were isolated in 1976 from benomyl sprayed orchards (Pelser, 1977). Unfortunately, subsequently resistance monitoring was not reported scientifically, although the presence of IMZ resistant strains of *P. digitatum* was reported from all South African citrus growing regions (CRI Annual Report, 2008).

The highest registered dose of IMZ applied through conventional methods (4000 $\mu g.g^{-1}$ in a wax formulation) may not load enough residue to sufficiently control sporulation of green mould caused by IMZ-resistant biotypes of *P. digitatum* (Eckert et al., 1994). However, in heated IMZ dip treatments, the albedo of wounds took up significantly more IMZ compared to unwounded albedo at levels that should be able to control most IMZ resistant strains (Dore et al., 2009). Cross-resistance and multiple resistance to combinations of IMZ, TBZ and SOPP have been reported (Bus et al., 1991; 1992; Holmes and Eckert, 1999; Kinay et al., 2007).

In order to control the development of resistance, (Bancroft et al., 1984) recommended sanitation and disinfection of the contaminated areas, isolation of the inoculum source of resistant strains, and to have a monitoring program for fungicide resistance in packhouses. Due to the fact that IMZ-resistant strains of *P. digitatum* did not compete well with their sensitive counterparts it was concluded that IMZ-resistant strains will not survive in the field in the absence of IMZ selection pressure (Dave et al., 1989). In California, Urugay and Marocco, no IMZ resistant isolates of *P. digitatum* have been found in the orchards and IMZ-resistance

was mainly found in the packhouses (Holmes and Eckert, 1999; Kinay et al., 2007; Boubaker et al., 2009; Pérez et al., 2011).

Azoxystrobin, fludioxonil and pyrimethanil are three new fungicides with alternative modes of action and were introduced during the mid 2000's to the American postharvest industry for use against citrus green mould. These fungicides can play an important role in IMZ resistance management (D'Aquino et al., 2006; Smilanick et al., 2006; Kanetis et al., 2008; Schirra et al., 2010; D'Aquino et al., 2013). Resistance to pyrimethanil has, however, been reported of isolates from orchards in California (Kinay et al., 2007). Unlike azoxystrobin and fludioxonil, pyrimethanil does not have a comparable ability to inhibit sporulation in comparison to IMZ (Smilanick et al., 2006; Kanetis et al., 2007). Although a combination of imazalil and pyrimethanil were able to significantly inhibit green mould sporulation caused by IMZ-sensitive isolates it could not effectively inhibit sporulation in IMZ-resistant isolates (Kanetis et al., 2007). However this combination is able to effectively control green mould development caused by IMZ- and TBZ-resistant isolated of *P. digitatum* (Kanetis et al., 2007).

4. Conclusion

Dispite decades of research the green mould pathogen, *Penicillium digitatum*, is still the major cause of losses due to decay. Imazalil (IMZ) is currently the most effective and popular fungicide applied in citrus packhouses worldwide. Temperature, the fungicide carrier (water or wax), pH, exposure time and application method all play a role in loading the optimum residue on the fruit as well as green mould control. Prior to the start of this study, the South African situation around IMZ application and green mould control was not clear, especially the impact of IMZ resistance.

5. Objectives

From the literature research presented here, the following objectives were highlighted for this study:

- Assess IMZ application methods in the South African industry by conducting a survey of all factors that could impact residue loading and green mould control.
- Investigate the optimisation of IMZ residue loading and subsequent disease control through laboratory trials on a variety of citrus types.
    - Include the influence of IMZ concentration, solution pH, solution temperature and exposure time.
    - Assess alternative application methods such as drench and spray.
    - Investigate the effect of post dip brushing, a general packhouse treatment, on residue loading en disease control.
- Investigate the curative ability of IMZ on different citrus types
- Determine the IMZ residue required for effective curative and protective control of a selection of IMZ sensitive and resistant *P. digitatum* and *P. italicum* isolates.
- Assess alternative fungicides for their ability to curatively and protectively control IMZ resistant isolates.

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CHAPTER 2[a]

IMAZALIL RESIDUE LOADING AND GREEN MOULD CONTROL IN CITRUS PACKHOUSES

Abstract

Imazalil (IMZ) is commonly applied in South African citrus packhouses for the control of green mould, caused by *Penicillium digitatum*, yet the disease still causes significant postharvest losses. Maximum residue limit (MRL) for IMZ on citrus fruit is 5 $\mu g.g^{-1}$, whereas 2-3 $\mu g.g^{-1}$ is regarded as a biologically effective residue level that should at least inhibit green mould sporulation. Standard compliance auditing of residue levels of citrus fruit, however, indicated that fruit from the majority of packhouses have residues of ≈ 1 $\mu g.g^{-1}$. Poor disease control from insufficient residue loading might further be compounded by the presence of IMZ-resistant isolates of *P. digitatum* in packhouses. This study was conducted to assess the current status of IMZ application in South African packhouses, to determine the adequate residue levels needed to control green mould and inhibit its sporulation using both IMZ sensitive and resistant isolates, to investigate IMZ application methods and resultant residue levels in commercial citrus packhouses, and to study optimisation of modes of IMZ application in citrus packhouses. Factors studied were IMZ concentration, application type (spray *vs.* dip and drench), exposure time, solution temperature and pH, as well as curative and protective control of *P. digitatum*. The packhouse survey showed that the majority of packhouses applied IMZ in a sulphate salt formulation through a fungicide dip tank, and loaded an IMZ residue of ≈1 $\mu g.g^{-1}$. In dip applications, IMZ had excellent curative and protective activity against *Penicillium* isolates sensitive to IMZ. However, curative control of IMZ resistant isolates was substantially reduced and protective control was lost, even at twice the recommended concentration, nor was sporulation inhibited. The use of sodium bicarbonate (2%) buffered imazalil sulphate solutions at pH ±8, compared with pH ±3 of the unbuffered solutions, markedly increased IMZ residue loading on Navel and Valencia oranges and improved curative and protective control of IMZ resistant isolates. Exposure time did not affect IMZ residue loading in IMZ sulphate solutions at pH 3, although the MRL was exceeded after 45 s exposure in pH 8 solutions. Imazalil applied through spray or drench application improved residue loading, but green mould control was less effective than after dip application.

1. Introduction

Imazalil (IMZ) is applied in citrus packhouses for the control of *Penicillium digitatum* (Pers.:Fr.) Sacc. (Laville et al., 1977; Eckert, 1995), which causes green mould (Smith, 1897). This post-harvest disease is a major cause of decay on citrus fruit globally (Eckert and Eaks, 1989). Various application methods of IMZ are possible. One such possibility is the pre-packline drenching of fruit bins shortly after harvest. In the

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packline it can be applied through several options: a fungicide dip tank, spray, drench or mixed with wax in wax application systems (Laville et al., 1977; McCornack et al., 1977; Kaplan and Dave, 1979). Combinations of these methods can also be done, where double or even triple application of IMZ is possible. In the early 1980s, a dip treatment for 1 to 3 min in 250 – 500 $\mu g.mL^{-1}$ IMZ was recommended as the most effective application method and IMZ sulphate was recommended as the preferred formulation due to its water-solubility (Pelser and La Grange, 1981). Currently IMZ is available in two different formulations. Imazalil sulphate, which is formulated as soluble granules or powders and has a very good ability to dissolve in water. It is therefore recommended in aqueous application at 500 $\mu g.mL^{-1}$. The IMZ emulsifiable concentrate (EC) is an oily emulsion of IMZ and is mostly recommended for incorporation in wax applications at 3000 $\mu g.mL^{-1}$.

Smilanick et al. (2005) showed that the effectiveness of IMZ to control green mould was positively correlated with the pH level of the IMZ solution. They found that IMZ residue measured on orange fruit dipped for 30 s in a 500 $\mu g.mL^{-1}$ IMZ EC solution at pH 7 or 9 was two-fold of that measured on fruit dipped in solutions at pH 3 or 5. In another test they found that amending a 500 $\mu g.mL^{-1}$ IMZ solution with 3% $NaHCO_3$ did not significantly affect residue loading on the fruit, although the addition of the $NaHCO_3$ markedly improved the effectiveness of the IMZ to control green mould and affected partial control of IMZ resistant isolates of the pathogen. In Australia, it was also indicated that the efficacy of IMZ was improved when mixed with either 0.5 or 3% $NaHCO_3$ (Cunningham and Taverner, 2006). Recently, Dore et al. (2010) confirmed these findings showing that $NaHCO_3$ increased IMZ residues in wounds, but not intact fruit. Prior work employed the IMZ EC formulation, which has a relatively neutral pH and limited water solubility, and not that of IMZ sulphate, which has a low pH and is very water soluble. It is not known how the IMZ sulphate would perform in similar trials. It is particularly important to know the influence of $NaHCO_3$ on fruit IMZ residues, since compliance with regulator tolerances has become increasingly important, and its influence on IMZ performance, since IMZ resistant isolates of *P. digitatum* are common.

Smilanick et al. (1997) found that IMZ solution temperature, concentration and exposure time all have an effect on the subsequent loading of IMZ residues on fruit. Warmer temperatures, higher concentrations and longer exposure times increased the IMZ residue on treated fruit. By using the EC formulation of IMZ, they found the ideal combination was a solution temperature of 37.8ºC, at a concentration of 350 to 400 $\mu g.mL^{-1}$ and an exposure time of 30 s. An IMZ residue of 2 to 4 $\mu g.g^{-1}$ on fruit can be expected. Schirra et al. (1997) found that dipping lemon fruit in a much lower IMZ concentration, *i.e.* 50 $\mu g.g^{-1}$, but at longer exposure (3 min) and higher solution temperature (50ºC) loaded residue levels of ≥ 2 $\mu g.g^{-1}$ and gave significant protection against citrus green mould.

In earlier studies, atomisers were used to apply IMZ, either in water or in water based wax solutions over rotating brushes, and adequate residue levels were obtained; however, IMZ applied in water gave better results in terms of residue loading and green mould control (Brown et al., 1983; Brown, 1984). It was also stated that application of IMZ in water will also give more protection against infections occurring after treatment (Brown, 1984).

The potential to develop fungicide resistance was shown in a group of fungi, which included *P. digitatum* (van Tuyl, 1977). Resistance to IMZ is polygenic and involves 21 genes on 8 loci and is linked with 6 groups; this means that it is theoretically more difficult for *Penicillium* to develop resistance due to all the different genes involved (Laville et al., 1977). However, resistance did eventually develop (Holmes and Eckert, 1995). Imazalil is a demethylation inhibitor of the biosynthesis of ergosterol, and resistance was

affected primarily by overexpression of cytochrome P450-dependent sterol 14 alpha-demethylase (Ghosoph et al., 2007). Infection caused by resistant isolates cannot be controlled or its sporulation inhibited with normal packhouse IMZ treatments (Eckert et al., 1994). A higher IMZ residue will improve green mould control and combat IMZ resistance development (Dore et al., 2009). IMZ resistance has been reported in South Africa (Jacobs and Korsten, 2010), but the extent and level is not known.

The purpose of this study was firstly to conduct a survey in South African packhouses in order to determine the most commonly used IMZ application methods and their efficacy in terms of IMZ residue loading. Secondly, the most commonly used IMZ application method was studied and compared to other methods in terms of residue loading and control of resistant and sensitive isolates of *P. digitatum*.

2. Materials and methods

2.1. Survey of commercial IMZ application in South African citrus packhouses

Current fungicide application methods were evaluated by a survey of South African packhouses. Detailed surveys of the various pack lines included IMZ concentration, solution pH and temperature, fruit exposure time and IMZ fruit residue analyses.

The temperatures of the dip tank solutions in the operating packhouses were measured by means of thermo probe connected to an infrared thermometer (Sentry ST642; Sentry Optronics Corporation, Shanghai, China). The exposure time of the fruit to the dip tank solution was measured by wrapping some of the fruit in aluminium foil and measuring the exposure time of these marked fruit in the dip tank; this was replicated 12 times. A 250-mL sample of the dip tank solution was taken if IMZ was part of the solution. The samples were stored at -20°C until the IMZ concentration of each was determined by a two-phase titration method (Janssen Pharmaceutica, Beerse, Belgium). Sodium lauryl sulphate (90-91%, UniLab, Krugersdorp, South Africa) was titrated into 25 mL of the IMZ solution to which 10 mL sulphuric acid (Synthon, Nijmegen, Netherlands), 25 mL dichloromethane (99.8%, Sigma-Aldrich, St Louis, Missouri, USA) and 12 drops of indophenol blue indicator (Sigma-Aldrich, St Louis, Missouri, USA) had been added. When the end point was reached the amount of sodium lauryl sulphate (mL) used was noted as “A”. The same procedure was followed with a blank solution of water and the end point (mL) was noted as “B”. The IMZ concentration ($\mu g.mL^{-1}$) was calculated as (A mL – B mL) × 0.1 × 40 × 297.18. The pH of the solutions was measured by means of a pH meter (Jenway Model 3310; Bibby Scientific Limited, Staffordshire, UK).

Six fruit were sampled before and after each IMZ application on a specific packline. The sampled fruit were deep frozen (-20°C) until prepared for IMZ residue analysis. The fruit were defrosted, measured and weighed and macerated to a fine pulp by using a blender (Salton Elite, Almalgamated Appliance Holdings Limited, Reuven, South Africa) and re-frozen. Sub-samples of the macerated fruit were submitted for IMZ (chloramizol) residue analyses by Hearshaw and Kinnes Analytical Laboratory, Cape Town, South Africa. The samples were extracted using acetonitrile followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography mass spectrometry mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, California, USA). The data collected were subjected to descriptive and Pearson correlation statistics using SAS (SAS Institute Inc. Cary, NC, USA).

2.2. IMZ residue loading and biological efficacy trials

IMZ residue loading after simulated dip, spray and drench application systems and curative and protective application for the control of green mould was studied in several experiments.

2.2.1. Isolates, IMZ sensitivity and storage

An IMZ-resistant (R) isolate of *P. digitatum* was obtained from Citrus Research International (CRI) in Nelspruit, South Africa and an IMZ-sensitive (S) isolate was obtained from a Satsuma orchard on the Stellenbosch University experimental farm, Welgevallen, Stellenbosch, South Africa. The species identity of the two isolates was confirmed as being *P. digitatum*, since BLAST analyses in GenBank showed that (1) the ITS sequence of both isolates had 100% similarity to the *P. digitatum* sequence (AY373910) of (Haugland et al., 2004) and (2) the β-tubulin sequence of both isolates had 99.8% similarity to the *P. digitatum* sequence (AY674405) of Samson et al. (2004). Sensitivity to IMZ was determined by Janssen Pharmaceutica, Beerse, Belgium. In brief, potato dextrose agar was amended with an IMZ concentration range of 10 to 0.001 $\mu g.g^{-1}$ on which spore suspensions of the tested isolates were inoculated. Cultures were incubated for 7 days at 22°C before the growth was measured and compared to the control (no IMZ). EC50 and EC95 values were calculated by linear interpolation in a XY-plot with a logarithmic concentration scale by using the growth diameter values of cultures with 50 and 5% growth of the control. The respective EC50 and EC95 values for the S-isolate (STEU6560) were 0.07 and 0.10 $\mu g.mL^{-1}$ and for the R-isolate (STEU6590) they were 1.83 and 4.52 $\mu g.mL^{-1}$. The isolates were stored in the culture collection of the Department Plant Pathology, University Stellenbosch, South Africa.

In order to obtain inoculum for biological efficacy tests, the isolates were grown at ambient temperature on potato dextrose agar (PDA) medium in Petri dishes and were re-plated in 2-week cycles. Conidia were harvested from 10- to 14-day-old cultures approximately 1 hour before trials commenced. The surface of a culture was washed with sterile deionised water amended with Tween 20 (Sigma-Aldrich, St Louis, Missouri, USA) added at a concentration of 0.01 $mL.L^{-1}$ into a 500 mL Schotts bottle to a volume of 100 mL. The conidial suspensions were amended to a concentration of 1×10^6 $spores.mL^{-1}$ by means of a haemocytometer. The conidial suspensions were placed on magnetic stirrers to maintain a homogenised suspension of spores.

2.2.2. Fruit

Untreated export quality citrus fruit were collected from various citrus packhouses in the Western Cape province of South Africa. Citrus type and cultivar used for specific trials varied in accordance to seasonal availability. Before the trials commenced fruit were stored at 3.5 - 7°C for ±3 days. A day before a trial, fruit were transferred from cold storage to ambient in order for fruit temperature to reach ambient and to allow any possible condensation to evaporate.

2.2.3. Inoculation

Fruit were treated before inoculation (protectively) or after inoculation (curatively) to evaluate the efficacy of IMZ treatment dosages to control S and R isolates of *P. digitatum*. The fruit for the curative treatment were inoculated 4 to 6 hours before treatment with IMZ. Fruit were wounded with a triple wound inducer, which consisted of three insect needles placed in a needle clamp to create three small wounds of 0.5 mm wide and 2 mm deep at a triangular distance of 1.5 mm apart. Wounding and inoculation were

conducted simultaneously by dipping the wound inducer into a spore suspension of *P. digitatum* (1×10^6 spores.mL^{-1}) immediately prior to wounding. Four wounds were induced on each fruit at equal distances around the stem-end; 12 fruit were inoculated per treatment with three replications. Fruit for protective treatment were first treated with IMZ, left to dry and inoculated as above before placed with the curatively treated fruit in the specific storage regimes.

2.2.4. Imazalil and residue analysis

In the various experiments conducted, fruit were treated with the imazalil sulphate formulation (Imazacure, 750 g.kg^{-1} SG, ICA International Chemicals, Stellenbosch, South Africa), unless stated differently. IMZ sulphate dissolved in municipal water and at concentrations of 250 to 1000 µg.mL^{-1} resulted in a solution pH of ≈3. For certain treatments, 2% sodium bicarbonate ($NaHCO_3$; Alkalinity Plus, Pool Perfect, Bellville, South Africa) was applied to IMZ sulphate solutions to increase the pH to ≈8 in an attempt to improve residue loading (Smilanick et al., 2005). A fresh treatment solution was prepared for each treatment. Six fruit per treatment were sampled and frozen (-20°C) until prepared for IMZ residue analysis as described previously. Two separate samples were analysed for each treatment.

2.2.5. Fruit storage and evaluation

The 12 treated and inoculated fruit from each treatment combination were packed in lock back table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd, Atlantis, South Africa). Each carton was covered with a transparent polyethylene bag and sealed with a cable tie. Different storage regimes were used in the various experiments and will be discussed**.** At the end of storage, the percentage of infected fruit was determined and sporulation was evaluated for each fruit by means of a rating index of 0 to 6, where 0 = no sign of disease, 1 = lesion visible but no sporulation, 2 = sporulating area on lesion smaller than a quarter of the fruit, 3 = sporulating area larger than a quarter of the fruit, but smaller than half of the fruit, 4 = sporulating area larger than half of the fruit, but smaller than three quarters of the fruit, 5 = sporulating area larger than three quarters of the fruit, but smaller than the whole fruit and 6 = sporulating area covering the whole fruit. Sporulation incidence (%) was determined from infected fruit with a sporulation index of 2 and higher. The incidence of infection (%) was derived from the sporulation ratings, where fruit were regarded as infected if they had a sporulation index of higher than 0.

2.2.6. Statistical analysis

Imazalil residue data were continuous and analysed by an analysis of variance appropriate to the experimental layout. Binomial infection incidence and sporulation data were transformed to percentages and working logits before subjected to an appropriate analysis of variance using statistical software (SAS version 9.2, SAS Institute Inc. Cary, NC, USA). Shapiro-Wilk test was performed to test for non-normality (Shapiro and Wilk, 1965). In cases where non-normality was due to high kurtosis, analysis was conducted on percentages (Glass et al., 1972). In the case of non-normality due to skewness, logit transformed data were used. Student's t-Least Significant Difference was calculated at the 5% significance level to compare treatment means. The experiments (on the different citrus types) were analysed separately, but if variances between citrus types were of the same magnitude as determined by Levene's test, the data were combined (John and Quenouille, 1977). If heterogeneity of variances were found a weighted analysis of variances was

done. Weights were calculated by using the reciprocal of the experimental variance (mean square error) for each experiment. In the combined analysis this led to a mean square error of 1 (MSE=1).

2.3. IMZ application in a dip tank

2.3.1. Effect of IMZ concentration

Oranges (*Citrus sinensis* (L.) Osbeck) were immersed for 60 s in IMZ concentrations 0, 250, 500 and 1000 µg.mL^{-1} at pH ≈ 3 or 8 (amended with 2% $NaHCO_3$) at a solution temperature of ±20°C and left to dry at ambient temperature. A plastic container (330 × 620 × 285 mm) with 20 L of each specific solution was used as IMZ dip tank. Fruit were treated before inoculation (protectively) or after inoculation (curatively) with the S or R isolate of *P. digitatum* as described previously. Two storage regimes were followed after treatment; one at 7°C for 21 days plus a subsequent 7 days at 23°C (simulating the cold chain for export fruit) and another at 23°C for 14 days. Evaluations were conducted 11, 14, 18 and 21 days after treatment for fruit stored at 7°C, and after the additional 7 days at 23°C. For fruit stored at 23°C, evaluations were conducted 2, 4, 7, 11 and 14 days after treatment. Sporulation and infection incidences were determined as described earlier.

Three replications of 12 fruit per treatment were used. For each treatment, two of the three replications had six additional fruit for residue analysis. The trial was done twice, once with navel and once with Valencia oranges.

2.3.2. Effect of exposure time and solution temperature

Valencia oranges (2 replications of 6 fruit per treatment combination) were immersed for 15, 45, 90, 180 or 540 s in the registered concentration of 500 µg.mL^{-1} IMZ solution that was not amended or amended with 2% $NaHCO_3$ at either 20 or 35°C. After treatment fruit were left to dry at ambient before it was deep frozen and prepared for residue analysis as previously described. A temperature-controlled stainless steel water bath (Unitemp Water Bath, Baird and Tatlock (London) Ltd., Essex, UK) was used as an IMZ dip tank.

2.3.3. IMZ dip application compared to spray application

By measuring deposition of a fluorescent pigment, Fourie et al. (2009) demonstrated significantly higher deposition values with spray volumes to the point of run-off, compared with sprays past run-off and dip applications. In order to ascertain whether point-of-run-off IMZ sprays to citrus fruit will result in better residue loading than dip treatments, an experiment was conducted to compare spray application with dip treatment. Eureka lemons (*Citrus limon*) inoculated with the IMZ S and R isolates were treated curatively in this experiment. For the dip tank application, fruit were immersed for 60 s in a 0, 250, 500 and 1000 µg.mL^{-1} IMZ solution (20°C), which was not amended (pH 3) or amended with 2% $NaHCO_3$ (pH 8), or in a IMZ EC (Imazacure 500 EC, ICA International Chemicals, Stellenbosch, South Africa) solution at the same concentrations. For spray application, the point of run-off was determined using the methods described by Fourie et al. (2009). A gravity-fed mist spray gun (ITW DEVILBISS Spray Equipment Products, Glendale Heights IL 60139 USA) was used to apply the spray volumes to fruit at a pressure of 1 bar. Spraying was done in a spray chamber [660 × 1410 × 880 mm (h×l×w)] with individual fruit positioned directly below the spray gun, which was mounted onto the spray chamber at a distance of 60 cm from the fruit. A spray volume of 3 mL of IMZ sulphate, alone (pH ≈ 3) or amended with 2% $NaHCO_3$ (pH ≈ 8), was applied at

concentrations of 0, 1000, 2000 and 4000 µg.mL^{-1} and IMZ EC at the same concentrations. Three replications of 12 fruit per treatment combination were treated. An additional 6 fruit (not inoculated) were treated in each of two replications for residue analysis. After each dip or spray treatment, the fruit were left to dry at ambient before fruit were sampled for residue analysis. The remaining fruit were stored at 20°C for 14 days to evaluate infection and sporulation). The trial was repeated on Valencia oranges.

2.4. IMZ application in a drench

Mineola (Tangelo Mineola) fruit inoculated with the IMZ S and R isolates were treated curatively in this experiment. Commercial bin-drenching systems were simulated by using plastic fruit crates (280 × 480 × 310 mm) with 20 × 20 mm diameter holes drilled in the bottom of the plastic crates to simulate the floor-openings in a commercial bin. Three crates were stacked on top of each other to represent a commercial fruit bin stack. The sides of the crates were closed with plastic sheets to ensure that the solutions used for drench treatment would flow from the top, through the middle and bottom crates into an empty collection crate in the bottom. Non-inoculated fruit were placed at the bottom and sides of each experimental crate. In three replications, 24 inoculated fruit, 12 × R and 12 × S, plus six non-inoculated fruit for IMZ residue analyses were placed in the middle of each crate. The inoculated fruit were placed at different angles in relation to the position of the inoculated sites around the stem end: four of the 12 fruit inoculated with a specific isolate were placed on their sides, 4 with the stem end facing upward and 4 with the stem end downward. The 3-crate stacks were drenched with 6.25, 12.5, or 25.0 L of a 500 µg.mL^{-1} IMZ EC solution at ambient temperature. These three volumes related respectively to 125, 250, or 500 L of the IMZ solution running through a stack comprising three commercial fruit bins. The control was treated with 12.5 L water containing no IMZ. Approximately 15 min after each application the experimental stack was disassembled and left overnight to dry. The inoculated fruit were packed in cartons as described previously and stored for 14 days at 20°C before being evaluated for green mould decay and sporulation as described previously. The trial was repeated on Eureka lemons and Valencia oranges.

3. Results

3.1. Survey of commercial IMZ application in South African citrus packhouses

During the 2008 season, 37 packhouses were visited throughout South Africa. Packhouses applied IMZ once only (46%), as a double application (49%), or even as three separate applications (5%). In the survey it was found that 78.4% of the packhouses use a dip tank to apply IMZ as a single application (38%), as a dip- and wax-application (38%), or as a pre-packline drench-, dip- and wax-application (3%). IMZ application through waxing systems was also common (62.2%), often in combination with the bath and pre-packline drench as mentioned previously, but also a single application (8%), or with inline drench (3%) and total loss spray systems (3%). Table 1 shows all the parameters measured on the dip tank during the survey. Dip tank capacity and length varied from 1000 L and 1 m to 8000 L and 10 m, respectively, with the majority containing ≈2500 L at a length of 2.5 m. Fruit exposure time in the dip tank solution was positively correlated with dip tank length (r^2 = 0.57) and varied from 15.5 to 106.8 s with the median at 47.1 s; only 25% of the packhouses had an exposure time longer than 60 s. Dip tank solution temperature varied from 11.5 to 44.6°C with the median at 33.4°C and the pH varied from 3.3 to 8.0 with the median at 5.4. The pH of the

solution was negatively correlated to its IMZ concentration (r^2 = -0.67) and ranged from 131.0 to 2175.4 µg.mL^{-1} with the median at 362.6 µg.mL^{-1}; and the 25th percentile was <250.00 µg.mL^{-1}. The dip tank solution age (days from starting with fresh solution) at time of assessment varied from 1 to 40 days with the median being 5 days old. After each dip tank, in every packhouse surveyed, a number of brushes were installed to reduce excess water before the fruit entered a wind tunnel for drying prior to wax application. The number of brushes after the dip tank varied from 8 to 52 with 20 brushes as the median. The IMZ residue measured on fruit that went through the different dip tanks varied from 0.2 to 3.9 µg.g^{-1} with the median at 1.0 µg.g^{-1}. IMZ residue level was poorly correlated with exposure time (r^2 = -0.16), pH (r^2 = 0.16), temperature (r^2 = 0.18) and IMZ concentration (r^2 = 0.20) in/of the solution.

3.2. IMZ application in a dip tank

3.2.1. Effect of IMZ concentration

Analysis of variance for IMZ residue data as determined on Navel and Valencia fruit treated in different concentrations of IMZ sulphate at either pH 3 or 8 showed no significant interaction involving citrus type (P > 0.05; ANOVA not shown), nor was citrus type significant as main effect (P = 0.981); data for citrus type were therefore combined. IMZ concentration and treatment (IMZ sulphate at pH 3 or 8) showed a significant interaction (P = 0.0006). Mean residue levels loaded on Navel and Valencia fruit in 0, 250, 500 or 1000 µg.mL^{-1} IMZ sulphate concentration at pH 3 increased from 0.09 µg.g^{-1} to 0.51 µg.g^{-1} to 0.97 µg.g^{-1} to 1.45 µg.g^{-1} (Table 2). The concomitant increase in the same concentrations of IMZ sulphate at pH 8 was from 0.03 µg.g^{-1} to 1.43 µg.g^{-1} to 3.50 µg.g^{-1} to 11.20 µg.g^{-1}, respectively.

Analysis of variance of infection incidence data obtained at the various evaluation times showed no significant and/or meaningful interaction involving evaluation time (ANOVA not shown). Green mould infection data at the end of the incubation periods of the different incubation regimes, *i.e.* day 14 of fruit stored at 23°C (ambient), day 21 of fruit stored at 7°C (cold storage) and after the 7-day shelf period (shelf life) for the cold stored fruit, were therefore analysed. Analysis of variance of these data showed a significant interaction between IMZ concentration, isolate (S or R), treatment action (curative or protective) and solution pH (P = 0.0006; ANOVA not shown), as well as for citrus type, IMZ concentration, isolate and incubation regime (P = 0.0022).

Fruit that were treated 4-6 h after inoculation, *i.e.* curatively, had lower levels of infection when compared to the protectively treated fruit (Table 3). In the control treatments (0 µg.mL^{-1}), fruit treated curatively had significantly less infection than those treated protectively (43.6% *vs.* 67.7% for the S isolate 58.3% *vs.* 73.0% for the R isolate). The S isolate caused significantly less decay than R on the control fruit in the curative treatment, but not in the protective treatment (Table 3). The addition of 2% $NaHCO_3$ to water reduced infection significantly in the curative treatment (24.5% and 37.7% for the R and S isolate, respectively) but not in the protectively treated fruit. On fruit treated with 250 µg.mL^{-1} IMZ sulphate at pH 3 (residue of 0.51 µg.g^{-1}; Table 2) the S isolate caused significantly lower levels of decay when treated curatively (2.5%; Table 3) and protectively (12.8%). This residue level on fruit also significantly reduced the R isolate infection (22.1%), but not when treated protectively (69.1%). The 0.97 µg.g^{-1} IMZ residue loaded with the 500 µg.mL^{-1} IMZ at pH 3 (Table 2) could curatively control the S isolate better than protectively (0% *vs.* 7.4%; Table 3), although infection incidences were not significantly different. On these fruit, the R isolate was significantly better controlled curatively than protectively (17.2% *vs.* 71.1%). A similar IMZ residue of

1.45 and 1.43 µg.g^{-1} were loaded on fruit treated with 1000 µg.mL^{-1} IMZ sulphate and 250 µg.mL^{-1} IMZ sulphate at pH 8, respectively (Table 2). These treatments had a significantly better curative than protective effect of the R isolate (8.3 – 11.8% *vs.* 45.6 – 52.0%, respectively; Table 3), which was not evident for the S isolate as it was almost completely controlled by both curative and protective treatment (< 2%). The residue was increased from 0.97 µg.g^{-1} to 3.50 µg.g^{-1} when the pH of the 500 µg.mL^{-1} IMZ sulphate solution was increased from 3 to 8 (Table 2). The S isolate was completely controlled by this treatment, curatively and protectively, while the R isolate was controlled significantly better with the pH 8 IMZ solution in the curative (7.4%; Table 3) and protective (19.1%) treatment. The 11.20 µg.g^{-1} IMZ loaded with the 1000 µg.mL^{-1} IMZ sulphate at pH 8 (Table 2), which was more than double the MRL of 5 µg.g^{-1}, reduced R infection levels to <7.0% when treated curatively or protectively (Table 3).

Table 4 summarises the significant interaction involving citrus type, IMZ concentration, isolate, and incubation regime. Navel oranges were generally more sensitive to green mould than Valencia oranges with significantly higher levels of infection recorded for the S and R isolate on control fruit (0 µg.mL^{-1}) following all incubation regimes, except for the S isolate after cold storage. The highest decay levels on control fruit were observed following the ambient incubation regime (>80% on Navel fruit and >50% on Valencia fruit; Table 4). Following cold storage of Navel fruit containing no IMZ, infection levels increased significantly after the 7-day shelf life period: from 31.3% to 60.4% and 52.1% to 64.6% for the S and R isolate, respectively. This was also evident for Valencia fruit, although not at significant levels (33.3% to 43.1% and 38.2% to 47.2% for the S and R isolate, respectively). The above-mentioned differences in decay levels on control fruit that followed the various incubation regimes was also evident on IMZ treated fruit, although significant only for the R isolate on Navel fruit. Decay caused by the S isolate on IMZ treated fruit ranged from 0 to 8.3%, while significantly higher levels were recorded for the R isolate on Navel (9.7 to 65.5%) and Valencia fruit (6.3 to 34.7%). Generally, R isolate infection levels were significantly lower after IMZ treatment on Navels after cold storage plus shelf life when compared to the ambient incubation period (39.6, 25.1 and 18.8% *vs.* 65.6, 49.3 and 41.0%, respectively for the 250, 500 and 1000 µg.mL^{-1} IMZ treatments). A similar trend was observed for Valencia, but it was in most cases not significant.

Due to the effective control of the S isolate by most IMZ treatments, sporulation incidence and severity data were too sparse for statistical analysis. The total inhibition of sporulation on infected fruit was rarely observed regardless of treatment or isolate. However, when the frequency distribution of sporulation index values was considered, a clear distinction was observed between the S and R isolate. Irrespective of IMZ treatment, sporulation index values on decayed R-inoculated fruit mostly (65%) exceeded a rating of 3 and often obtained a rating of 6. Decayed S-inoculated fruit were mostly (54%) rated at index values 1 to 3, while this frequency was even lower on fruit treated with higher IMZ concentrations (results not shown).

3.2.2. Effect of exposure time and solution temperature

The analysis of variance for residue data on Valencia fruit dipped for various lengths of time in a 20 or 35°C IMZ sulphate solution (500 µg.mL^{-1}) at pH 3 or pH 8 showed no significant interactions among exposure time, temperature, and pH (P = 0.6847; ANOVA not shown). Significant two-factor interactions were observed for exposure time and pH (P = <0.0001), while temperature showed no significant effect (P > 0.6). Exposure time had no significant effect on residue loading in the IMZ sulphate solutions at pH 3 and residues ranged from 1.22 (15 s) to 2.06 µg.g^{-1} (540 s) (Figure 1). Exposure time significantly affected IMZ residue loading in the IMZ sulphate solution at pH 8 where residue levels increased in a linear trend from

3.86 (15 s) to 44.41 µg.g^{-1} (540 s). These levels were significantly higher than those obtained in the pH 3 IMZ solutions from an exposure time of 90 s onward. However, the MRL level of 5 µg.g^{-1} was already exceeded at 45 s in the pH 8 IMZ sulphate solution.

3.2.3. IMZ dip tank application compared to spray application

Analysis of variance of the IMZ residue levels measured on Eureka lemon and Valencia fruit showed significant interactions among application method (dip or spray), IMZ concentration, and IMZ solution applied (*i.e.* IMZ sulphate at pH 3 or 8 and IMZ EC (P = 0.0010; ANOVA not shown). Very low levels of IMZ residue (0.05 to 0.18 µg.g^{-1}; Table 5) were detected on the fruit from the control dip or spray treatments. The registered concentration (1000 µg.mL^{-1}) for IMZ sulphate sprayed loaded more than double the amount of IMZ on the fruit compared to the registered dosage (500 µg.mL^{-1}) for IMZ sulphate applied in a dip tank (6.03 and 2.39 µg.g^{-1}, respectively;). When IMZ sulphate solution at 500 µg.mL^{-1} was buffered with 2% $NaHCO_3$ at pH 8, the IMZ residue level loaded following dip application (8.00 µg.g^{-1}) was comparable to that of the 1000 µg.mL^{-1} spray application of IMZ sulphate (6.03 µg.g^{-1}). IMZ residue loaded on fruit sprayed with or dipped in the registered concentrations of IMZ EC for the specific applications (1000 and 500 µg.mL^{-1}, respectively) was statistically similar although markedly higher for the dip application (5.16 µg.g^{-1} *vs.* 3.08 µg.g^{-1}). Sprays with the IMZ EC formulation generally resulted in lower residue levels than the IMZ sulphate (pH 3 or 8) sprays. In most cases double the registered concentration more or less doubled the residue loaded when compared to those loaded from the registered dosages with the only exception being IMZ sulphate solution at pH 3 sprayed where the increase was just over half the residue of the registered dosage, from 6.03 to 9.86 µg.g^{-1}. Furthermore, all these treatments (double the registered dosage) loaded a residue of higher than the MRL (5 µg.g^{-1}) except for the 1000 µg.mL^{-1} IMZ sulphate dip at pH 3, which loaded 4.82 µg.g^{-1}.

Analysis of variance furthermore showed a significant citrus type × application × treatment interaction (P = 0.0135; ANOVA not shown). Lemon fruit generally loaded more IMZ across all treatments (2.47 – 11.32 µg.g^{-1}) compared to Valencia (1.97 – 8.01 µg.g^{-1}; results not shown).

For infection data, analysis of variance showed significant 4-factor interactions involving citrus type (P < 0.05; ANOVA not shown), which were largely ascribed to significantly higher mean decay levels on Valencia than on lemon fruit after dip or spray treatments with 0 µg.mL^{-1} (93.1% *vs.* 19.4% and 66.7% *vs.* 94.4%, respectively; results not shown). Interactions with citrus type were therefore ignored. Significant 3-factor interactions among application method (dip or spray), treatment (IMZ sulphate at pH 3 or 8 and IMZ EC) and IMZ concentration (0, 250, 500 or 1000 µg.mL^{-1}) and among application method, IMZ concentration, and isolate (S and R) were found (P < 0.05), but the interactions among IMZ action, IMZ solution, IMZ concentration, and isolate (P = 0.2628) will be discussed in order to demonstrate the effects of these four factors. As shown in Table 6, decay levels caused by the S and R isolates were generally similar after the dip or spray control treatments (0 µg.mL^{-1}). Inoculated fruit dipped in water had significantly lower levels of infection (<63.0%) when compared to the water sprayed fruit (>77.0%). Addition of 2% $NaHCO_3$ alone significantly reduced infection levels in these treatments (dip and spray) to between 47.3 and 59.8%. In general, the various IMZ treatments yielded statistically similar decay levels, whether applied as dip or spray. However, residue levels loaded by spray treatments were significantly higher in the case of the IMZ sulphate solution at pH 3, and similar in the cases of IMZ sulphate at pH 8 and IMZ EC solutions, although 4 times lower concentrations were used in the dip treatments (Table 5). The 2.39, 8.00 and 5.16 µg.g^{-1} loaded, respectively, by the registered IMZ concentration of 500 µg.mL^{-1} dip treatment of the IMZ sulphate at pH 3,

IMZ sulphate at pH 8, and IMZ EC solutions reduced decay levels to 4.2, 0.0 and 1.4%, respectively, for S isolate infection (Table 6). These IMZ residue levels reduced the respective R isolate infection to 16.7, 8.3 and 7.0%. The registered concentration (1000 $\mu g.mL^{-1}$) to spray IMZ loaded residues of 6.03, 4.37 and 3.08 $\mu g.g^{-1}$ (Table 5), which reduced S isolate infection to 4.2, 1.4 and 1.4%, respectively (Table 6). This range of IMZ residue levels reduced R isolate infection to 20.9, 15.4 and 27.9%, respectively; this was significantly higher than the S isolate infection. Decay levels by the R isolate on IMZ treated fruit were generally higher than that of the S isolate; significantly at the lower treatment concentrations.

As mentioned previously, data for sporulation on infected fruit were sparse given the complete control of the S isolate after IMZ treatment and were therefore not statistically analysed. Total inhibition of sporulation was again rarely observed. Generally higher sporulation index values were recorded on decayed fruit inoculated with the R isolate. Sporulation index values rated for decayed S-inoculated fruit also declined with increasing IMZ concentration and similar index values was recorded on decayed dipped and sprayed fruit (results not shown).

3.3. IMZ application in a drench

Analysis of variance for IMZ residue levels measured in the drench trial showed a significant interaction between citrus type (Mineola, Lemon and Valencia) and volume (P = <0.0001; ANOVA not shown). Crate stacking as main effect was not significant (P = 0.6922). Lemon fruit loaded significantly more IMZ (2.92 – 3.90 $\mu g.g^{-1}$) across drench volumes when compared to Valencia (1.72 – 2.47 $\mu g.g^{-1}$) and Mineola (1.56 – 1.99 $\mu g.g^{-1}$) as shown in Table 7. When drenched with the 6.25 L, Mineola and Valencia fruit loaded similar IMZ residue levels (1.56 and 1.72 $\mu g.g^{-1}$, respectively) and this was significantly lower than that loaded on lemon fruit (2.92 $\mu g.g^{-1}$). When the 6.25 L volume was doubled, Mineola, Valencia and lemon fruit loaded significantly different levels of IMZ residue (1.65, 2.19 and 3.80 $\mu g.g^{-1}$, respectively); except for Mineola, this was significantly higher than loaded with the 6.25 L volume. Residue levels increased, but not to significantly higher levels, when fruit was drenched with 25 L.

Analysis of variance for the green mould infection data showed a significant interaction between isolate (S and R) and solution drench volume (P = <0.0001; ANOVA not shown). Citrus type as main effect was not significant (P = 0.4838), nor was crate stacking (P = 0.1794). As shown in Table 8, infection caused by the S isolate was significantly reduced from 91.7% (control) to 9.3, 6.0, or 1.9 when drenched with the IMZ EC volumes of 6.25, 12.50, or 25.00 L, respectively. Fruit inoculated with the R isolate had significant higher levels of infection (56.9 – 69.0%) compared to those inoculated with the S isolate across the three volumes. When the 12.5 L volume was doubled to 25 L the infection decreased for the R and S isolate from 65.3 to 56.9% and 6.0 to 1.9%, respectively, although these differences were not significant.

The percentage of sporulating infected fruit showed trends similar to the infection results: fruit inoculated with the R isolate had significant more sporulating infected fruit (74.9 – 80.3%, across the three volumes) compared to the infected fruit inoculated with the S isolate (16.7 – 39.6%, across the three volumes; results not shown). Complete sporulation inhibition could not be observed in any treatment, although there was a tendency for S isolate infected fruit to have lower sporulation index ratings when compared to the R isolate infected fruit across all treatments. The majority of the S isolate infected fruit had a sporulation rating of three or lower and no index ratings of six could be observed. The R isolate infected fruit showed the opposite results with the majority showing a sporulation rating of 3 and higher.

4. Discussion

From the packhouse survey it was evident that the majority of South African citrus packhouses apply IMZ in the sulphate formulation through fungicide dip tanks. However, considerable variation was observed in the application systems employed by the different packhouses. IMZ residue loaded to fruit was poorly correlated with any of the eight parameters measured in and around the fungicide dip tank, thus indicating a complex interaction between these factors and IMZ residue loading following dip treatment with the IMZ sulphate formulation. In research mostly using the EC formulation of IMZ, concentration, solution temperature and pH, exposure time, and brushes after aqueous treatment have been shown to have an individual or combined effect on IMZ residue loading (Eckert, 1977; Cabras et al., 1999; Schirra et al., 1996, 1997; Smilanick et al., 1997, 2005; D'Aquino et al., 2006; Dore et al., 2009, 2010).

Commercial residue results obtained during 2008 and 2009 show that South African citrus packhouses loaded a median IMZ residue of ≈ 1 $\mu g.g^{-1}$ (data not shown) on fruit and this is reflected in the survey. The ideal residue attained from aqueous treatment of IMZ is regarded to be around 2 $\mu g.g^{-1}$ in order to effectively control green mould or at least inhibit sporulation (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997). Inadequate residue loading will result in loss of disease control and development of IMZ resistance in packhouses, highlighting the need to study means of improving IMZ residue loading and green mould control.

Previous studies on IMZ application and the subsequent residue loading were predominantly done with the emulsifiable concentrate formulation of IMZ (IMZ EC). Due to the extensive use of the IMZ sulphate formulation in South Africa and the fungicide dip tank it required thorough investigation. Methods to optimise IMZ residue loading were explored. Statistically, IMZ concentration had no significant effect on residue loading in fruit treated with IMZ sulphate at pH 3, although IMZ residues increased with increments of ≈ 0.50 $\mu g.g^{-1}$ as treatment concentration was increased from 250 to 500 to 1000 $\mu g.mL^{-1}$. After 60 s in a 500 $\mu g.mL^{-1}$ IMZ sulphate dip tank (registered concentration for fungicide dip tanks in South Africa; pH 3) a residue of 0.97 $\mu g.g^{-1}$ was loaded on oranges. These findings concur with work done by Smilanick et al. (2005) where oranges dipped for 30 s in a 500 $\mu g.mL^{-1}$ of an IMZ EC solution at a pH of 3 loaded a residue of <1.00 $\mu g.g^{-1}$. This residue is >1.00 $\mu g.g^{-1}$ lower than the ideal level to sufficiently control green mould (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997), but relates with what is found on fruit treated in commercial packhouses. The suboptimal residue currently loaded on citrus through the fungicide dip tank in South African packhouses could be ascribed to the formulation and its properties, which will be discussed below.

An IMZ residue of ≈ 1 $\mu g.g^{-1}$ could control 4 – 6 h old green mould infections of an S isolate of *P. digitatum*. Infections by an R isolate were significantly reduced, but control was not complete. The incubation time in this study for the curative treatments was short and does not simulate the worst-case scenario in industry, where fruit generally stand for ≈2 days before treatment. Overall it was clear that IMZ applied through a dip tank has better curative than protective action against green mould. Dore et al. (2009) also demonstrated better curative control on lemons dipped in IMZ EC, with better curative and protective control on fruit treated in a 50°C compared with 25°C solution.

IMZ residue loading was improved when the dip tank solution of IMZ sulphate was buffered with 2% $NaHCO_3$ to pH 8. Smilanick et al. (2005) showed that when the pH of a mixture of 500 $\mu g.mL^{-1}$ IMZ EC and 3% $NaHCO_3$ were decreased from 7 to 3, the residue loaded on oranges was reduced from ≈ 2 $\mu g.g^{-1}$ to ≈ 1

$\mu g.g^{-1}$, but a pH increase from 7 to 9 did not influence residue loading. In our study, increasing the pH of the 500 $\mu g.mL^{-1}$ IMZ sulphate formulation from 3 to 8 resulted in a 3-fold increase in residue level, and with the 1000 $\mu g.mL^{-1}$ IMZ sulphate the increase was more than 7-fold. In both cases, this led to complete curative or protective control of the S isolate. The control of the R isolate infections was also improved, but not complete. Addition of 1 to 3% $NaHCO_3$ to an IMZ EC solution led to improved green mould control compared to IMZ EC alone and a synergy between IMZ and $NaHCO_3$ was suggested (Dore et al., 2010; Smilanick et al., 2005). In the case of IMZ sulphate combined with $NaHCO_3$ a possible synergistic effect could have been masked by the markedly increased residue loading and needs further study.

Long term cold storage (21 days at 7°C) had an adverse effect on green mould infection, especially on the R isolate, and in the majority of cases IMZ increased this effect. The control treatments had up to 20% less infection on fruit after the cold storage plus shelf life incubation regime when compared to fruit incubated at ambient. To our knowledge, this phenomenon has not been described in literature and our observations might be explained by the relatively short incubation time and small wounds used in our study. Curatively treated fruit were placed in cold storage 8 to 10 hours after inoculation and treatment at 23°C, while protectively treated fruit were incubated 2 to 4 hours after inoculation. By this time, the majority of conidia would have germinated (Plaza et al., 2003) and the infection process started. Optimal growth and germination takes place at 25ºC and growth of *P. digitatum* is retarded at temperatures below 10ºC (Kassim and Khan, 1996; Lacey, 1989; Plaza et al., 2003). Therefore at 7°C the growth of germinated and the germination of conidia will be retarded and so too the inoculum potential (Garrett, 1958). Wounds induced in this study were 2 mm deep, which should be just below the flavedo. Wounds at this depth should be more resistant to infection than deeper wounds often used in other studies (Kavanagh and Wood, 1967). Proportionally more wound healing might also have taken place during cold storage, as well as the deposition of wound-induced material (Stange et al., 1993; Mulas et al., 1996; Lai et al., 2003). The effect of wound healing can be increased during the shelf-life period (Mulas et al., 1996). Nonetheless, it was clear from this study that cold storage exhibited limited levels of curative control of recent infections of *P. digitatum*. This observation provides an additional reason, other than delaying senescence, for rigorous attention to proper temperature management of the fruit after harvest.

Solution temperature at ambient (± 20°C) and 35°C had no significant influence on residue loading for fruit dipped in either IMZ sulphate solution at pH 3 or pH 8. This is in contrast with IMZ EC formulation where temperature had a positive effect on IMZ residue loading (Schirra et al., 1996; Smilanick et al., 1997). IMZ residues loaded on Navel oranges increased more than 4-fold when a 410 $\mu g.mL^{-1}$ IMZ sulphate solution temperature was increased from 21.1 to 40.6°C (Smilanick et al., 1997). The effect of temperature on IMZ residue loading through the sulphate salt formulation needs to be investigated further.

Buffering of the IMZ sulphate solution with 2% $NaHCO_3$ at pH 8 significantly increased the residue loaded over time in comparison with IMZ sulphate at pH 3. Commercial IMZ sulphate is actually a bisulphate salt that consists of equimolar quantities of IMZ base (1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy-ethyl]-1 *H*-imidazole and sulphuric acid (H_2SO_4) (Siegel and Ragsdale, 1978). Dissolution of the salt in water establishes an equilibrium mixture of different hydrated ion species; these are the protonated IMZ base and hydronium cations, as well as hydrosulphate and sulphate anions. The pK_a value of IMZ is 6.5 (Siegel et al., 1977), which is the pH in water at which 50% of dissolved IMZ molecules will be protonated and 50% will be unprotonated. Sulphuric acid is a strong diprotic acid that has both its pK_a values below 2.0. Therefore in the equilibrium mixture of IMZ bisulphate in water the strong acid species will predominate causing the pH to

be about 3 and virtually all IMZ molecules will be protonated and hydrated. On addition of a base such as $NaHCO_3$, the strong acids will be neutralised, thereby changing the composition of the equilibrium mixture and increasing the solution pH. Every change of a single pH unit above the pK_a of IMZ (6.5) will cause an order of magnitude increase in the molar concentration of IMZ base (unhydrated water insoluble) and a concomitant decrease in the protonated IMZ cations (hydrated, water soluble). Use of neutralised solutions of IMZ sulphate buffered at pH above 7 is not recommended. Based on the general principles of partition coefficients it should be expected that the hydrophobic base of IMZ will readily and preferentially dissolve in the waxy/oily cuticle of the citrus fruit. When fruit is dipped into aqueous IMZ bisulphate (pH 3) we have shown that the transfer of IMZ to the fruit rind is a slow process and may even have an upper limit (<2.10µg.g^{-1} after 540 s). It is speculated that IMZ may be transferred by deprotonation of IMZ cation by basic components on the fruit surface, followed by dissolution of the liberated lipophilic IMZ base in the oils of the cuticle. In contrast, transfer of IMZ from the pH 8 IMZ sulphate solution to the rind is much faster (>3.00 µg.g^{-1} after 15 s). The IMZ sulphate solution at pH 3 can therefore be regarded as a safe application in terms of the MRL of 5 µg.g^{-1}. Other results from our study support this argument. In the exposure trials, a fresh dip tank solution of 500 µg.mL^{-1} IMZ sulphate at pH 3 loaded an IMZ residue of 1.53 µg.g^{-1} after 45 s exposure time. Even under these controlled conditions, the residue level was below 2 µg.g^{-1}. When the pH was increased to 8 by means of 2% $NaHCO_3$ the residue loading was increased significantly; after 45 s it was well above the MRL of 5 µg.g^{-1} and can therefore not be recommended for commercial use. The limited solubility of IMZ sulphate at pH 8 also makes it unsuitable for practical recommendation. Further research is required to establish whether increased residue loading can be attributed to the buffer ($NaHCO_3$) or the pH level. Concomitantly, the effect of exposure time in the IMZ sulphate solution on the control of green mould also needs to be investigated.

Spray application of aqueous IMZ solutions onto fruit to just before the point of runoff (Fourie et al., 2009) improved residue loading when compared to dip application. Spray application with the registered dosage (1000 µg.mL^{-1}) loaded a mean of 6.03 µg.g^{-1} and the 60 s dip application with the registered dosage (500 µg.mL^{-1}) loaded 2.39 µg.g^{-1}. However, this higher IMZ residue did not improve control of either the R or the S isolate of *P. digitatum* and dip application resulted in superior curative control, even at significantly lower IMZ residue levels. This could possibly be attributed to hydraulic pressure assisting IMZ residue loading into wound sites (Brown, 1984) when applied through the dip tank. The EC spray application loaded lower IMZ residue levels onto fruit, which can be attributed to increased levels of run-off observed at the spray volumes used (Fourie et al., 2009). Other researchers have also found the spray application of IMZ EC to be less effective in terms of green mould control when compared to dip applications (Kaplan and Dave, 1979; Brown and Dezman, 1990).

By drenching a simulated 3-crate stack of fruit bins with 250 to 500 L of 500 µg.mL^{-1} IMZ EC residue levels of 1.65 to > 2 µg.g^{-1} were loaded on fruit. However, the R isolate could not be controlled in any volume or on any citrus type with the infection level >50% across treatments. The S isolate was reduced to <10% infection for the simulated 250 and 500 L drench treatments. It has been shown that field bin drenches with thiabendazole (TBZ) significantly increased control of a TBZ resistant isolate, although the infection level was close to 60% (Smilanick et al., 2006). Degreening conditions (48 to 72 hours at high humidity and mild temperatures) are conducive to green mould development and it will be extremely risky to protect fruit with sub-lethal residue levels of IMZ and even TBZ on fruit. This will only enhance and encourage the development of resistance. The drench application system needs to be studied further with

the aim to improve green mould control on fruit during the degreening process.

A very important attribute of IMZ is its ability to inhibit sporulation (McCornack and Brown, 1977). However, the ability of IMZ to inhibit sporulation was not clearly evident from this study. There were indications of this ability on the S isolate infections with sporulation ratings mostly ≈ 3, but sporulation inhibition of the R isolate was rarely observed with ratings mostly > 3. The reason for the absence of sporulation inhibition in this study could be due to the inoculation method and incubation period of 4 – 6 h. In studies where sporulation inhibition was specifically examined a spore suspension of *P. digitatum* was injected into the core of the fruit before treatment with IMZ (Brown et al., 1983; Brown and Dezman, 1990).

Overall the different citrus types responded in similar trends to IMZ residue loading and green mould control. Eureka lemons consistently loaded higher IMZ residues levels compared to other citrus types, Valencia oranges and Mineola soft citrus. This tendency for lemons to load higher residues was shown with regards to thiabendazole (Schirra et al., 1997), but it was regarded not to be the case for IMZ (Cabras et al., 1999). Valencia oranges tended to be more resistant to green mould than Navel oranges, although similar IMZ residues levels were loaded. In another case Eureka lemons showed more resistance to green mould than Valencia oranges.

The IMZ sulphate formulation, about which little was known until our work, reacts rather differently in terms of residue loading following dip, drench, or spray applications compared to the well-studied IMZ EC formulation. Increasing levels of post-harvest green mould and/or loss of sporulation inhibition on fruit packed and exported from South Africa can be attributed to IMZ resistance development and sub-optimal IMZ treatment and residue loading in packhouses. This study shows the benefit of increased residue loading following addition of $NaHCO_3$ or increasing the pH of the dip solution. This aspect needs to be investigated further to allow its safe and practical implementation.

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Table 1. Descriptive statistics of parameters measured in and around fungicide dip tank of packhouses that use this method to apply imazalil (IMZ) sulphate

	n	Min	1st quartile	Median	3rd quartile	Max
Capacity (L)	25	1000	2000	2501	3400	8000
Length (m)	26	10	2	2.5	4.2	10
Temperature (°C)	28	11.5	26.5	33.4	37.3	44.6
pH	28	3.34	4.6	5.4	6.8	8.0
Time (s)	28	15.5	24.7	47.1	64.2	106.8
IMZ concentration ($\mu g.mL^{-1}$)	22	131	249.6	362.6	615.2	2175.4
Age (d)	19	1	4	5	8	40
Brushes	27	8	12	20	27	52
Residue ($\mu g.g^{-1}$)	29	0.24	0.89	1.02	1.70	3.85

Table 2: Mean imazalil (IMZ) residue levels measured on Valencia and Navel oranges dipped for 60 s in different concentrations of IMZ sulphate at pH 3 and 8 (buffered with 2% $NaHCO_3$)

	IMZ residue ($\mu g.g^{-1}$)[a]	
Concentration ($\mu g.mL^{-1}$)	**IMZ sulphate (pH 3)**	**IMZ sulphate (pH 8)**
0	0.09c	0.03c
250	0.51c	1.43bc
500	0.97bc	3.50b
1000	1.45bc	11.20a

[a]Means followed by the same letter do not differ significantly ($P < 0.05$; LSD = 2.875)

Table 3. Mean green mould infection incidence (%) on Valencia and Navel oranges inoculated with either an imazalil (IMZ) sensitive or resistant isolate of *P. digitatum*, that were dipped either curatively (after 4-6 h incubation) or protectively (inoculated after treatment) for 60 s in a range of IMZ sulphate concentrations (0 – 1000 µg.mL^{-1}) at pH 3 and 8 (buffered with 2% $NaHCO_3$) after ambient (14 days at 23°C) and cold-stored incubation periods (21 days at 7°C plus an additional 7 days at 23°C)

	Green mould infection incidence (%)[a]			
	IMZ sulphate (pH 3)		IMZ sulphate (pH 8)	
IMZ concentration (µg.mL^{-1})	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**
Curative				
0	43.6ef	58.3cd	24.5g	37.7f
250	2.5j	22.1g	1.0j	11.8hi
500	0.0j	17.2gh	0.0j	7.4ij
1000	0.0j	8.3ij	0.0j	5.9ij
Protective				
0	67.7ab	73.0a	61.3bc	65.2abc
250	12.8hi	69.1ab	1.0j	45.6ef
500	7.4ij	71.1a	0.0j	19.1gh
1000	2.0j	52.0de	0.0j	6.9ij

[a]Means followed by the same letter do not differ significantly (P = 0.05; LSD = 0.09)

Table 4. Mean green mould infection incidence (%) caused by an imazalil (IMZ) sensitive or resistant isolate of *P. digitatum* on Valencia and Navel oranges dipped for 60 s in a range (0 – 1000 µg.mL^{-1}) of IMZ sulphate concentrations at pH 3 and 8 (buffered with 2% $NaHCO_3$) after ambient (14 days at 23°C) and cold-stored incubation periods (21 days at 7°C plus an additional 7 days at 23°C)

	Green mould infection incidence (%)[a]			
	Navel		Valencia	
IMZ concentration (µg.mL^{-1})	Sensitive	Resistant	Sensitive	Resistant
14 days at 23°C				
0	81.3a	84.7a	50.0cde	66.7b
250	6.9nop	65.6b	8.3mnop	34.7ghi
500	4.2op	49.3de	3.5op	35.4ghi
1000	0.7op	41.0efgh	2.1op	22.9jk
21 days at 7°C				
0	31.3hij	52.1cd	33.3ghij	38.2fgh
250	0.7op	25.0ijk	0.0p	25.0ijk
500	0.0p	20.1kl	0.0p	15.3klmn
1000	0.0p	9.7lmnop	0.0p	6.3nop
21 days at 7°C + 7 days at 23°C				
0	60.4bc	64.6b	43.1defg	47.2def
250	7.3nop	39.6efgh	3.5op	33.3ghij
500	4.2op	25.1ijk	0.0p	25.7ijk
1000	0.0p	18.8klm	0.0p	11.1lmno

[a]Means followed by the same letter do not differ significantly (P = 0.05; LSD = 0.10)

Table 5. Mean imazalil (IMZ) residue levels measured on Valencia oranges and Eureka lemons dipped for 60s in or sprayed with 3 mL of various concentrations (0 – 1000 or 4000 µg.mL^{-1}, respectively) of IMZ sulphate at pH 3 or 8 (buffered with 2% $NaHCO_3$) or the IMZ EC formulation.

IMZ concentration (µg.mL^{-1})	IMZ residue (µg.g^{-1})[a]		
	IMZ sulphate (pH 3)	IMZ sulphate (pH 8)	IMZ EC
Dip			
0	0.05i	0.10i	0.05i
250	1.03hi	2.67ghi	2.94fghi
500	2.39ghi	8.00bcd	5.16defg
1000	4.82efg	18.34a	9.39b
Spray			
0	0.18hi	0.11i	0.18hi
1000	6.03cde	4.37efg	3.08fgh
2000	9.86b	8.69bc	5.73def
4000	19.03a	17.71a	10.78b

[a]Means followed by the same letter do not differ significantly (P = 0.05; LSD = 2.924)

Table 6: Mean green mould infection incidence (%) after 14 days of storage at 20°C rated on Valencia oranges and Eureka lemons inoculated with imazalil (IMZ) sensitive and resistant isolates of *P. digitatum*, incubated for 4-6 hours and thendipped for 60s in or sprayed with 3 mL of various concentrations (0 – 1000 or 4000 µg.mL^{-1}, respectively) of IMZ sulphate at pH 3 or 8 (buffered with 2% $NaHCO_3$) or the IMZ EC formulation.

IMZ concentration (µg.mL^{-1})	Green mould infection incidence (%)[a]					
	IMZ sulphate (pH 3)		IMZ sulphate (pH 8)		IMZ EC	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Dip						
0	62.7b	50.2bc	51.5bc	47.3c	62.7b	50.2bc
250	4.2hij	48.6c	1.4j	18.1defg	0.0j	23.7de
500	4.2hij	16.7defgh	0.0j	8.3fghij	1.4j	7.0ghij
1000	0.0j	5.6ghij	0.0j	2.8ij	1.4j	1.4j
Spray						
0	77.9a	83.4a	58.5bc	59.8bc	77.9a	83.4a
1000	4.2hij	20.9def	1.4j	15.4defghi	1.4j	27.9d
2000	0.0j	22.3de	2.8ij	20.9def	0.0j	12.6efghij
4000	1.7j	8.4fghij	0.0j	15.4defghi	0.0j	5.6ghij

[a]Means followed by the same letter do not differ significantly (P = 0.05; LSD = 13.0)

Table 7: Mean imazalil (IMZ) residue levels (µg.mL^{-1}) measured on Mineola soft citrus, Eureka lemons and Valencia oranges drenched with different volumes (6.25 – 25.00 L) of 500 µg.mL^{-1} IMZ EC.

		IMZ residue (µg.g^{-1}) [a]		
Drench volume (litre)	**IMZ concentration (µg.mL^{-1})**	**Mineola**	**Lemon**	**Valencia**
12.50	0	0.00g	0.06g	0.02g
6.25	500	1.56f	2.92b	1.72ef
12.50	500	1.65ef	3.80a	2.19cd
25.00	500	1.99de	3.90a	2.47c

[a]Means followed by the same letter do not differ significantly (P = 0.05; LSD = 0.409)

Table 8: Mean green mould infection incidence (%) rated on Mineola soft citrus and Valencia oranges after 14 days of storage at 20°C inoculated with imazalil (IMZ) sensitive and resistant isolates of *P. digitatum*, incubated for 4-6 hand then drenched with different volumes (6.25 – 25.00 L) of 500 µg.mL^{-1} IMZ EC.

		Green mould infection incidence (%)[a]	
Drench volume (litre)	**IMZ concentration (µg.mL^{-1})**	**Sensitive isolate**	**Resistant isolate**
12.50	0	91.7 a	92.6 a
6.25	500	9.3 d	69.0 b
12.50	500	6.0 de	65.3 bc
25.00	500	1.9 e	56.9 c

[a]Means followed by the same letter do not differ significantly (P = 0.05; LSD = 0.48)

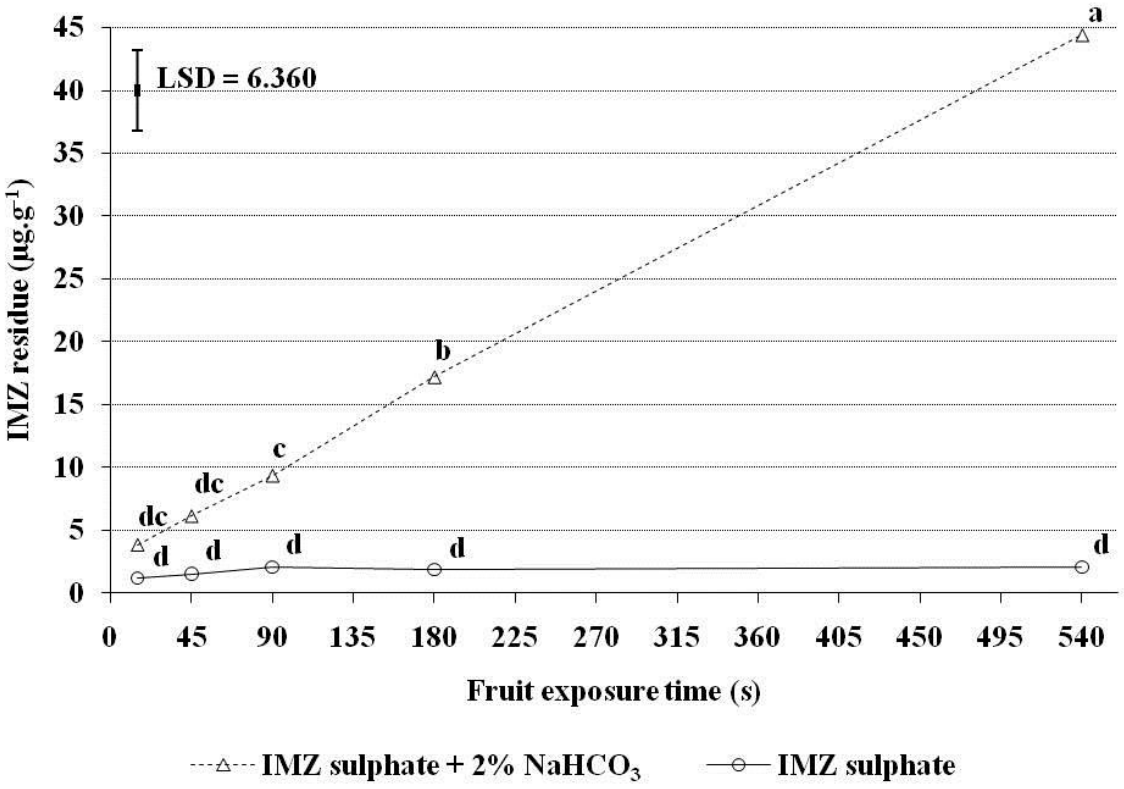

Figure 1. Mean imazalil (IMZ) residue levels measured on Valencia oranges dipped for various exposure times in a 500 µg.mL^{-1} solution (at 20 and 35°C, mean data presented) of IMZ sulphate at pH 3 or pH 8 (buffered with 2% $NaHCO_3$). Data points followed by the same letter do not differ significantly (*P* = 0.05; LSD = 6.360)

CHAPTER 3[a]

IMAZALIL RESIDUE LOADING AND GREEN MOULD CONTROL ON CITRUS FRUIT AS AFFECTED BY FORMULATION, SOLUTION pH AND EXPOSURE TIME IN AQUEOUS DIP TREATMENTS

Abstract

Green mould, caused by *Penicillium digitatum*, is responsible for major postharvest fruit losses on the South African fresh citrus export market. Some of these losses as well as fungicide resistance development can be attributed to sub-optimal imazalil (IMZ) residue loading on citrus fruit (<2 $\mu g.g^{-1}$), which is commonly the case in South African packhouses. This will result in loss of control and sporulation inhibition on decayed fruit. IMZ formulation [IMZ sulphate and emulsifiable concentrate (EC)], solution pH (IMZ sulphate at 500 $\mu g.mL^{-1}$ buffered with $NaHCO_3$ or NaOH to pH 6 and 8) and exposure time (15 to 540 s) were investigated in order to improve IMZ residue loading and the green mould control on Clementine mandarin, 'Eureka' lemon, and navel and Valencia orange fruit. Exposure time had no significant effect on residue loading in the unbuffered IMZ sulphate solution (pH 3). No differences were observed between the pH buffers used, but residue loading improved with increase in pH. The maximum residue limit (MRL) of 5.0 $\mu g.g^{-1}$ was exceeded following dip treatment in the IMZ EC (after 75 s exposure time), and IMZ sulphate at pH 8 using $NaHCO_3$ (77 s) or NaOH (89 s) as buffer. The MRL was exceeded after 161 s in IMZ sulphate solutions buffered at pH 6 with either $NaHCO_3$ or NaOH. An IMZ residue loading curve was prepared from which residue levels can be predicted for the control of IMZ-sensitive and IMZ-resistant isolates of *P. digitatum*. From this model the benchmark residue level for 95% control of an IMZ-sensitive isolate and of an IMZ-resistant isolate were predicted to be 0.81 and 2.64 ug g^{-1}, respectively. Residue loading can be improved by adjusting the pH level of an IMZ sulphate solution to 6 or by using the IMZ EC formulation, but exposure time should be restricted to 45 s so as not to exceed the MRL. Conversely, sufficient exposure time of ≈ 90 s in an unbuffered IMZ sulphate solution (pH 3) will result to improved green mould control, but with residue loading below 2 $\mu g.g^{-1}$. The resistant isolate could not be controlled adequately with residue levels below the MRL, therewith indicating the practical relevance of IMZ resistance.

1. Introduction

South African fresh citrus is exported to northern hemisphere destinations in the USA, Asia, the Middle and Far East, Europe, and Africa. Time from harvest to market is generally 4 weeks. Every year losses occur due to decay of which green mould, caused by the wound pathogen *Penicillium digitatum*

[a] Chapter published: Erasmus, A., Lennox, C.L., Smilanick, J.L., Lesar, K., Fourie, P.H., 2013. Imazalil residue loading and green mould control on citrus fruit as affected by formulation, solution pH and exposure time in aqueous dip treatments. Postharvest Biol. Technol. 77, 43-49.

(Pers.:Fr.) Sacc. (Smith, 1897), is usually the main contributor (Eckert and Eaks, 1989). Apart from sanitation and careful handling to prevent wounds, control relies primarily on postharvest fungicide applications.

Residue loading can give an indication of the effectiveness of fungicide application, but control of green mould can differ from one application method to another regardless of the specific residue level loaded (Erasmus et al., 2011 / Chapter 2). Although alternative application methods such as drench and sprays have shown promise in terms of residue loading, the fungicide dip tank applications gave better curative control and sporulation inhibition of green mould infections (Kaplan and Dave, 1979; Erasmus et al., 2011 / Chapter 2). In work done mainly with the IMZ EC formulation, exposure time(Brown and Dezman, 1990; Smilanick et al., 1997; Cabras et al., 1999), solution temperature (Smilanick et al., 1997; Cabras et al., 1999; Dore et al., 2009) and solution pH (Holmes and Eckert, 1999; Smilanick et al., 2005; Cunningham and Taverner, 2006; Dore et al., 2010) have all been shown to have an effect on IMZ residue loading on citrus fruits by means of a fungicide dip tank. Increasing the IMZ solution temperature alone can be used to control and increase residue loading, but the cost of heating may force packhouses to look at alternative ways to improve IMZ residue loading. Sodium bicarbonate ($NaHCO_3$) can improve the effectiveness of IMZ and residues loaded better at a higher pH (7 – 9) than at a lower pH (3 – 5) (Smilanick et al., 2005).

In South Africa, Pelser and La Grange (1981) recommended the IMZ sulphate formulation for dip application with a fruit exposure time of 1-3 minutes and at a concentration of 250 – 500 $\mu g.mL^{-1}$. This recommendation was followed widely and IMZ sulphate is currently applied extensively by 78% of South African citrus packhouses in a fungicide dip tank at 500 $\mu g.mL^{-1}$ (Erasmus et al., 2011 / Chapter 2). The emulsifiable concentrate (EC) formulation is applied primarily with wax in South Africa, but internationally it is also applied in aqueous dip or inline drench-type applications. A residue of 2 $\mu g.g^{-1}$ or higher is reported to give effective control or at least sporulation inhibition of green mould (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997). Erasmus *et al.* (2011 / Chapter 2) reported that during the 2007 to 2009 seasons the majority of South African packhouses loaded suboptimal residue levels of ≈1 $\mu g.g^{-1}$.

While dip exposure time in an IMZ sulphate solution had no significant effect on residue loading, increasing the solution pH with 2% $NaHCO_3$ from 3 to 8 increased IMZ residue loading by more than three-fold (Erasmus et al., 2011 / Chapter 2). These increased residue levels improved control of green mould caused by sensitive and resistant isolates. IMZ sulphate molecules dissolved in water are protonated and hydrated; as the pH is increased above the pKa of IMZ (6.5), the proportion of unhydrated (water insoluble) IMZ base molecules will increase. At a pH level of 8 the majority of IMZ molecules will precipitate over time, and can therefore not be recommended. The effect of pH levels lower than the pKa level of 6.5 had to be investigated in terms of residue loading and green mould control.

Due to the inadequate residue loading in South African packhouses, which often leads to inadequate control and loss of sporulation inhibition, the risk for resistance development is ever increasing. This and the extensive use of IMZ sulphate in fungicide dip tank applications highlight the need to optimise IMZ residue loading through this specific application. Increasing the pH level showed promise in earlier studies (Erasmus et al., 2011 / Chapter 2), but the effect of different buffers and pH levels needed to be investigated further to support a recommendation that would ensure optimal IMZ residue loading of > 2.0 $\mu g.g^{-1}$, without exceeding the maximum residue limit (MRL) of 5.0 $\mu g.g^{-1}$.

IMZ residue loading on various citrus types was studied in IMZ solutions with different pH levels and

exposure times. The IMZ sulphate formulation was also compared to the EC formulation in terms of residue loading. This work also presents a first attempt at determining the benchmark residue level required to successfully control IMZ sensitive and resistant isolates of *P. digitatum*.

2. Materials and methods

2.1. Isolates, IMZ sensitivity and storage

An IMZ sensitive (S) and a resistant (R) isolate of *P. digitatum* were used as described previously (Erasmus et al., 2011 / Chapter 2). The S isolate was sourced from the Welgevallen experimental farm of the University of Stellenbosch (Stellenbosch, South Africa) and the R isolate sourced from a South African packhouse by Citrus Research International (Nelspruit, South Africa). In order to obtain inoculum for biological efficacy tests, the isolates were grown at ambient temperature on potato dextrose agar (PDA; Biolab, Merck, Wadeville, Gauteng, South Africa) medium in Petri dishes and were re-plated in 2-week cycles. Conidia were harvested from 10- to 14-day-old cultures approximately 1 hour before trials commenced. The surface of a culture was washed with sterile deionised water amended with Tween 20 (Sigma-Aldrich, St Louis, Missouri, USA) at a concentration of 0.01 $mL.L^{-1}$. The conidial suspensions were amended to a concentration of 1×10^6 $spores.mL^{-1}$ by means of a haemocytometer. The conidial suspensions were placed on magnetic stirrers to maintain a uniform suspension of spores.

2.2. Fruit

Untreated export quality mandarin (cv. Nules Clementine), lemon (cv. Eureka), navel orange (cv. Washington) and Valencia sweet orange (cv. Midknight) fruit were obtained from various citrus packhouses in the Western Cape province of South Africa during the season of 2009 as the specific citrus types became available. Before trials commenced, fruit were stored at 3.5 - 7°C for ±3 days. A day before a trial, fruit were transferred from cold storage to ambient (± 20°C) in order for fruit temperature to reach ambient and to allow any condensation to evaporate.

2.3. *Imazalil and residue analysis*

Fruit were treated with an IMZ sulphate formulation (Imazacure, 750 $g.kg^{-1}$ SG, ICA International Chemicals, Stellenbosch, South Africa) or an IMZ EC (emulsifiable concentrate; Imazacure 500 EC, ICA International Chemicals, Stellenbosch, South Africa) formulation. For IMZ residue analyses, six fruit from two separate replicate treatments were sampled from the specific treatments described below, left to dry and frozen (-20°C) until prepared for IMZ residue analysis. The fruit were defrosted, weighed and then macerated to a fine pulp by using a blender (Salton Elite Blender, Almalgamated Appliance Holdings Limited, Reuven, South Africa) and re-frozen. Sub-samples of the macerated fruit were submitted for IMZ (chloramizol) residue analyses by Hearshaw and Kinnes Analytical Laboratory, Cape Town, South Africa. The samples were extracted using acetonitrile followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography mass spectrometry mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, California, USA). A fresh treatment solution was prepared for each treatment.

2.4. Effect of exposure time and pH on IMZ residue loading

Fruit were immersed for 15, 45, 90, 180 and 540 s in a 500 µg.mL^{-1} solution of either IMZ sulphate or IMZ EC. The pH of the IMZ sulphate solutions were adjusted to a pH of 6 or 8 by using either sodium bicarbonate ($NaHCO_3$; Alkalinity Plus, Pool Perfect, Bellville, South Africa) or sodium hydroxide (NaOH; Saarchem, Wadeville, South Africa). For $NaHCO_3$, 300 and 20000 mg.L^{-1} was added for pH6 and 8, respectively. For NaOH, ±90 and ±100 mg.L^{-1} was added for pH 6 and 8, respectively. A temperature-controlled stainless steel warm water bath (Unitemp, Baird and Tatlock Ltd., Essex, UK) was used as IMZ dip tank (24 L) and all solutions were kept at 35°C during treatments. Fruit were left to dry at ambient after treatment. For each treatment, samples of six fruit each per citrus kind were collected from two separate treatment repetitions for residue analysis.

Residue data following the different treatment combinations were subjected to linear regression statistics using statistical software (SAS version 9.2, SAS Institute Inc. Cary, NC, USA); intercepts of all lines were set at 0 (*i.e.* 0 µg.g^{-1} at 0 s dip). Analysis of variance was conducted and Student's t-Least Significant Difference was calculated at the 5% significance level to compare the slopes.

2.5. IMZ residue benchmarks for effective control of P. digitatum

Fruit were treated curatively and therefore inoculated 4 to 6 hours before IMZ dip-treatment with either the S or R isolates of *P. digitatum*. Fruit were wounded with a triple wound inducer, which consisted of three insect needles placed in a needle clamp to create three small wounds of 0.5 mm wide and 2 mm deep at a triangular distance of 1.5 mm apart. Wounding and inoculation were conducted simultaneously by dipping the wound inducer into a spore suspension of *P. digitatum* (1 × 10^6 spores.mL^{-1}) immediately prior to wounding. Four wounds were induced on each fruit at equal distances around the stem-end; 12 fruit were inoculated per treatment with three replications. In order to attain variable residue levels, inoculated fruit were dipped for 5, 15 and 90 s in a 250 µg.mL^{-1} and for 15, 90 and 540 s in a 500 µg.mL^{-1} solution of IMZ sulphate. The treatments were conducted in a temperature controlled stainless steel warm water bath at 35°C as previously described. Twelve fruit from each treatment combination were packed in lock back table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd, Atlantis, South Africa). Each carton was covered with transparent polyethylene bags and sealed with cable ties, thereby preventing any cross-contamination, but creating a relatively humid incubation chamber. Fruit were stored for 21 days at 3.5°C plus an additional 7 to 14 days at 20°C to simulate the standard cold chain for exported citrus fruit**.** At the end of storage, decay and sporulation were evaluated for each fruit as described by Erasmus *et al.* (2011; Chapter 2). Infected fruit were given a rating from 0 to 6 related to the fruit area covered with green sporulation: 0 = no infection, 1 = infection with no sporulation, 2 = sporulation area less than 25% of the fruit, 3 = sporulation area less than 50% of the fruit and more than 25%, 4 = sporulation area more than 50% of fruit and less than 75%, 5 = sporulation area more than 75% of the fruit and less 100%, and 6 = 100% covered with sporulating green mould. Sporulation incidence (%) was determined from infected fruit with a sporulation index of 4 and higher. The incidence of infection (%) was derived from the sporulation ratings, where fruit were regarded as infected if they had a sporulation index of higher than 0. For every citrus type the experiment was repeated 4 times in the same dip solutions per concentration. An additional 6 fruit each were treated with the first two repeats and stored for residue analyses as described earlier.

Imazalil residue data were analysed by analysis of variance appropriate to the experimental layout. Binomial infection incidence and sporulation data were transformed to percentages and working logits before

subjected to an appropriate analysis of variance using statistical software (SAS version 9.2, SAS Institute Inc. Cary, NC, USA). Shapiro-Wilk test was performed to test for non-normality (Shapiro and Wilk, 1965). In cases where non-normality was due to high kurtosis, analysis was conducted on percentages (Glass et al., 1972). In the case of non-normality due to skewness, logit transformed data were used. Student's t-Least Significant Difference was calculated at the 5% significance level to compare treatment means. The experiments (on the different citrus types) were analysed separately, but if variances between citrus types were of the same magnitude as determined by Levene's test, the data were combined (John and Quenouille, 1977). If heterogeneity of variances were found a weighted analysis of variances was done. Weights were calculated by using the reciprocal of the experimental variance (mean square error) for each experiment. In the combined analysis this led to a mean square error of 1 (MSE=1).

3. Results

3.1. Effect of exposure time and pH on IMZ residue loading

The R^2 values for the majority of the linear regression lines fitted to residue data measured on different citrus types (Clementine mandarin, 'Eureka' lemon, navel and Valencia orange) exposed for 15 to 540 s to various IMZ dip treatments were mostly > 0.90 (results not shown). Linear lines fitted poorly on the data for all the citrus types dipped in IMZ sulphate at pH 3 with R^2 of < 0.56, because IMZ residues changed little when the length of the dip was prolonged (results not shown).

Analyses of variance of the slopes of the linear regression lines showed a significant two factor citrus type × treatment interaction (P = 0.0123; ANOVA not shown). As shown in Table 1, regression lines for the different citrus types dipped in the unbuffered IMZ sulphate (pH 3) solution had similar slopes (0.004 - 0.005).

Slopes for regression lines fitted for the pH 6 treatments were generally significantly higher (0.022 to 0.040) than those for pH 3 treatments. Clementine mandarin, 'Eureka' lemon and navel orange fruit had similar slopes (0.030 – 0.040) in the IMZ sulphate pH 6 solutions regardless of the specific buffer. Regression lines for IMZ residue loading on Valencia orange fruit had lower slopes (0.025 and 0.022 for IMZ sulphate + $NaHCO_3$ and IMZ sulphate + NaOH, respectively) in the IMZ sulphate pH 6 solutions compared to the other citrus types. This difference was, however, only significant between Clementine mandarin (0.035 to 0.040) and Valencia orange fruit.

Slopes for regression lines fitted for the pH 8 treatments were generally significantly higher (0.044 to 0.074) than those for pH 6 and pH 3 treatments. On navel and Valencia orange fruit, the pH 8 slopes did not differ between buffers, but on Clementine mandarin and 'Eureka' lemon fruit the slopes were significantly higher (0.074 and 0.057, respectively) for the $NaHCO_3$ buffer, compared with NaOH (0.061 and 0.044, respectively). These differences were most likely the cause for the significant two factor interaction for citrus type and treatment (P = 0.0123; ANOVA not shown).

Slopes for the regression line fitted for IMZ EC treatment (0.050 to 0.084) was generally similar than those for the pH 8 treatments, but significantly higher than the pH 3 and 6 treatments. Clementine mandarin fruit dipped in the IMZ EC solution had the steepest slope (0.084) of all treatments. Navel and Valencia orange fruit had similar slopes (0.063 and 0.069, respectively), while 'Eureka' lemon fruit had a significantly lower slope (0.050) compared to the other IMZ EC treatments.

By disregarding the type × treatment interaction explained above, treatment as main effect, which was also significant in the analyses of variance (P = <0.0001; ANOVA not shown), can be explored further. Figure 1 shows the mean IMZ residue levels measured on the different citrus types exposed over the time intervals in the 500µg.mL^{-1} IMZ solutions as data points and the fitted linear regression lines over these data points. The slopes for fruit dipped in the unbuffered IMZ sulphate solution (0.0044) was significantly lower compared to all other treatments (0.0304 – 0.0668). Although the regression line fitted poorly (R^2 = 0.47; results not shown) for the unbuffered IMZ sulphate treatments, it showed the negligible effect of exposure time on this solution's residue loading ability. This is reflected in the average data for residue levels over time (1.55, 1.64, 1.74, 1.66 and 1.71 µg.g^{-1} loaded after 15, 45, 90, 180 and 540 s, respectively; Figure 1). The two IMZ sulphate pH 6 solutions had very similar loading trends in terms of slope (0.0311 and 0.0304 buffered with NaOH or $NaHCO_3$, respectively). The MRL of 5 µg.g^{-1} was exceeded after 161s. Slopes for the IMZ sulphate + $NaHCO_3$ pH 8 and IMZ EC treatments were similar (0.0668 and 0.0649, respectively), while the IMZ sulphate + NaOH pH 8 treatment had a significantly lower slope (0.0562). The MRL was exceeded after 75, 77 and 89 s exposure time in the respective solutions.

3.2. IMZ residue benchmarks for effective control of P. digitatum

Analysis of variance for IMZ residue data measured on various citrus types (Clementine mandarin, 'Eureka' lemon and navel and Valencia orange) showed that exposure time and treatment were significant as main effects (P <0.0001; ANOVA not shown). Citrus type was in no case significant; neither with the type × treatment interaction (P = 0.0829), nor as a main effect (P = 0.2490). Low IMZ residue levels (mean of 0.05 µg.g^{-1}, Table 2) were measured on fruit dipped for 60 s in water (with no IMZ; The low levels of IMZ (0.05 µg.g^{-1}) measured on fruit treated in water (0 µg.mL^{-1} IMZ) can be explained as contamination carried over during the analytical process (pers. comm. Sue Peal; Hearshaw and Kinnes Analytical Laboratory, Cape Town, South Africa). However, the fact that the S isolate reacted more severely compared to the R isolate point to the possibility that this contamination could have occurred during the trial, despite the thorough measures taken to clean the dip containers between treatments. Fruit dipped in 250 µg.mL^{-1} for 5, 15 or 90s loaded similar levels of IMZ residues (0.77, 0.82 and 0.79 µg.g^{-1}, respectively), but significantly higher than the 0 µg.mL^{-1} treatment. The 15 and 90 s treatments in 500 µg.mL^{-1} solution loaded similar levels (1.53 and 1.36 µg.g^{-1}, respectively), but significantly higher than those of the lower concentration. The 540 s treatment in the 500 µg.mL^{-1} loaded the highest IMZ level (1.66 µg.g^{-1}).

Analysis of variance for green mould infection incidence data showed a significant 2-factor treatment × isolate interaction (P = < 0.0001; ANOVA not shown). Infection levels from the S isolate on controls differed significantly; lower infection levels were observed following the 60 s water treatment (65.5%; Table 2), on which 0.05 µg.g^{-1} IMZ residue was measured, compared to the untreated control (91.0%). Nonetheless, infection was significantly reduced to 10.7% following dip-treatment for 5 s in 250 µg.mL^{-1}, which loaded a residue of 0.77 µg.g^{-1} (Table 2). Dip-treatment for 15 and 90 s in 250 µg.mL^{-1} (residue levels of 0.82 and 0.79 µg.g^{-1}, respectively) and 15 and 90 s in 500 µg.mL^{-1} (residue levels of 1.53 and 1.36 µg.g^{-1}, respectively) reduced S isolate infection to similar levels of between 2.4 and 3.9% (Table 2). Dips for 540 s in 500 µg.mL^{-1} (residue level of 1.66 µg.g^{-1}; Table 2*)* reduced the S infection to 1.1% (Table 2), which did not differ significantly from the previous mentioned ranges of treatments but differed significantly from the 10.7% on fruit treated for 5 s in 250 µg.mL^{-1}. The R isolate caused significantly lower levels of infection (79.3%) on the untreated fruit when compared to the S isolate (91.0%). This level of infection (79.3%) was slightly lower

(70.8%; not significantly) following the 60 s water treatment (Table 2). Compared to the untreated control (79.3%), IMZ treatment for 5, 15 and 90 s in 250 µg.mL^{-1} reduced infection levels significantly to 66.7, 66.5 and 62.4%, respectively (Table 2). The 15 and 90 s treatments in 500 µg.mL^{-1} IMZ sulphate lowered the R infection significantly to similar levels of 53.2 and 49.7%, respectively. This level was further significantly reduced to 36.0% by the 540 s treatment in 500 µg.mL^{-1}. All the R isolate inoculated IMZ treatments (250 and 500 µg.mL^{-1}) had significant higher levels of infection when compared with the same treatments inoculated with the S isolate.

The percentage control values of the S and R isolate infection ratings were plotted against the IMZ residues measured for each treatment. Cubic ($y = 73.194x^3 - 247.32x^2 + 269.64x$; $R^2 = 0.976$) and quadratic ($y = 7.5471x^2 + 16.082x$; $R^2 = 0.876$) polynomial trend lines were fitted for the S and R isolates, respectively. From these trend lines, 50 and 95% control levels of the S isolate were predicted at IMZ residue levels of 0.23 and 0.81 µg.g^{-1}, respectively. For the R isolate, 50 and 95% control levels were predicted at residue levels of 1.72 and 2.64 µg.g^{-1}, respectively.

Analysis of variance for green mould infection incidence data showed a meaningful citrus type × concentration × isolate interaction ($P = 0.1226$; ANOVA not shown). The interaction can be attributed largely to differences in infection by the S and R isolates on untreated fruit. Untreated Clementine mandarin fruit inoculated with either the S or the R isolate had equal levels of infection (95.8%; results not shown), while untreated navel orange fruit had similar levels of infection for the two isolates (91.7 and 95.8%, respectively). On Valencia orange fruit, the S isolate infection level was significantly higher compared to the R isolate (89.6 *vs.* 70.8%), as well as on 'Eureka' lemon fruit (85.4 *vs.* 45.8%).

Due to infection levels of 0% in the majority treatments with fruit inoculated with the S isolate, statistical analysis could not be done on sporulation ratings. Sporulation inhibition on infected fruit was generally not 100%, but certain trends were observed on the IMZ treated fruit. On Clementine mandarin fruit, an average of 10.4% was infected with the S isolate of which all showed sporulation inhibition (indexes of ≤ 3; results not shown). The average R infection level was 83.7% of which 22.3% to 45.0% showed sporulation inhibition; sporulation inhibition tended to increase with increasing treatment concentration. 'Eureka' lemon fruit had an average infection level for the S isolate of 2.1% and 18.4% for the R isolate. No sporulation inhibition was detected on fruit infected with the S isolate. On fruit infected with the R isolate, 10% and 25% showed sporulation inhibition for the 250 and 500 µg.mL^{-1} IMZ treatments, respectively. Navel and Valencia orange fruit had low levels of S isolate infections with an average of 1.4%. These infections were only observed in the lower treatment concentration (250 µg.mL^{-1}) and none of the navel orange fruit and all of the Valencia orange fruit showed sporulation inhibition. The R infection levels were 67.0% and 43.4% for navel and Valencia orange fruit, respectively. On IMZ treated navel fruit sporulation inhibition of the R isolate ranged from 24.5% to 43.2%. On Valencia orange fruit, sporulation inhibition of the R isolate ranged from 0% to 43.1%.

4. Discussion

The results from this study clearly showed the effect of IMZ formulation, solution pH and exposure time, as well as the interaction between these factors, on residue loading on citrus fruits. It is important to understand these interactions, as IMZ application in packhouses should be managed to ensure biologically

effective residue loading, while also adhering to maximum residue limits (MRL) as stipulated by food safety regulatory bodies (Hattingh and Hardman, 2011). Depending on the incubation criteria and the sporulation rating, researchers found that an IMZ residue of 1 – 2 $\mu g.g^{-1}$ (Kaplan and Dave, 1979; Dezman et al., 1986; Brown and Dezman, 1990; Eckert et al., 1994) or 2 – 3.5 $\mu g.g^{-1}$ (Smilanick et al., 1997) is necessary to control green mould or at least inhibit sporulation. In a survey of IMZ application, it was shown that South African packhouses load residues of ≈ 1 $\mu g.g^{-1}$ on fruit, mostly through aqueous dip applications of IMZ sulphate (Erasmus et al., 2011 / Chapter 2). Other studies have shown that IMZ residue loading could be improved by increasing the solution temperature, exposure time or IMZ concentration in aqueous dip applications (Brown and Dezman, 1990; Smilanick et al., 1997; Cabras et al., 1999; D'Aquino et al., 2006). However, these studies and others were done using the IMZ EC (emulsifiable concentrate) formulation (Eckert, 1977; Schirra et al., 1996, 1997; Smilanick et al., 2005; Dore et al., 2009; Dore et al., 2010).

Imazalil sulphate behaved differently to the IMZ EC formulation in aqueous dip applications (Erasmus et al., 2011 / Chapter 2). This was also demonstrated in the present study, as it was clear that exposure time had very little effect on residue loading in an unbuffered IMZ sulphate (pH 3) solution. However, when the pH was increased by means of $NaHCO_3$ or NaOH, a rapid increase in residue loading was observed. This rapid increase in residue loading was due to elevated pH levels and not the specific buffer, as little difference was observed between buffers used. An earlier study has also shown that elevating the pH level increased the activity of IMZ (Holmes and Eckert, 1999) and the combination of $NaHCO_3$ and IMZ EC significantly improved green mould control, as well as green mould caused by a resistant isolate (Smilanick et al., 2005). This combination, however, did not influence IMZ residue loading.

Erasmus et al. (2011; Chapter 2) offered a thorough explanation of the structure and behaviour of an aqueous IMZ sulphate solution. The product sold as IMZ sulphate is in reality IMZ bisulphate. IMZ has a pKa value of 6.5, indicating the pH level in an IMZ bisulphate solution where 50% of the dissolved IMZ molecules are protonated and 50% is unprotonated. When IMZ bisulphate is dissolved in municipal water (pH 7) to a concentration of 500 $\mu g.mL^{-1}$ the pH level decreases to < 3. This is due to the strong acidity of the sulphuric acid present in this solution, which is formed upon suspension of IMZ bisulphate in water (Erasmus et al., 2011 / Chapter 2). In this solution, the dissolved IMZ molecules are hydrated and protonated and in a more hydrophilic form. In this state the molecule is less available for absorption by the fruit rind. At pH 6, ≈50% of the dissolved IMZ molecules are protonated and ≈50% unprotonated. The protonated molecules will be hydrophilic and the unprotonated more hydrophobic. The unprotonated molecules should therefore be more available for absorption by the fruit rind. At pH 8, close to 100% of the dissolved IMZ molecules are unprotonated. In this solution IMZ is dissociated and residue loading will be rapid and high. Increasing the pH level of an IMZ sulphate solution to pH 6 showed promise as the ideal level of > 2 $\mu g.g^{-1}$ was reached after less than 45 s, which is the average fruit exposure time for South African citrus packhouse dip tanks (Erasmus et al., 2011 / Chapter 2). However, caution is advised as the MRL (5 $\mu g.g^{-1}$) was exceeded after 161 s in this solution. In IMZ sulphate solutions buffered to pH 8, residue loading was more rapid and the MRL was exceeded after 75 s. It should be noted that treatments in this study were commenced immediately after preparation of a fresh solution and continued for a limited time afterwards. This is not the case in a commercial packhouse situation where dip tank solutions are generally used 7 days, with regular top-up of the IMZ concentration. Using pH to improve IMZ residue loading might therefore be impractical or risky if management of IMZ sulphate dip application in commercial packhouses is not diligent. IMZ sulphate solutions at pH levels of ≥ 6.5 will also be with the practical problems of

precipitation and irregular distribution throughout the tank solution. Packhouses should also keep in mind that improved residue loading also affects the speed at which IMZ is stripped from the aqueous dip solutions, and the IMZ concentration should therefore be replenished accordingly.

The IMZ EC formulation had a similar residue loading pattern to IMZ sulphate solutions at pH 8. IMZ EC at the concentration used in this study is a very precarious option to use in the fungicide dip tank, as the MRL was exceeded with exposure times of ≥ 45 s at 35°C. It appeared as if a residue loading equilibrium was not reached, as a residue level of > 35.0 $\mu g.g^{-1}$ was loaded after 9 min. Our results in terms of IMZ EC residue loading were comparable to work done by others (Schirra et al., 1996; Smilanick et al., 1997, 2005; Cabras et al., 1999) with differences between studies ascribed to either solution temperature, exposure time or concentration.

Although exposure time had no effect on residue loading in unbuffered IMZ sulphate solutions (pH 3), there was evidence that exposure time had an effect on improved control of green mould. Similar residue levels were loaded, but infection levels of the S isolate were reduced almost three-fold when fruit was immersed for 90 s in the 250 $\mu g.g^{-1}$ IMZ sulphate solution compared to a 5 s dip. Even in the case of the R isolate, the difference was statistically significant between the 15 and 540 s 500 $\mu g.mL^{-1}$ treatments (53.2% and 36.0% infection, respectively). This observation indicates that IMZ residue levels should not be used as sole indicator of the effectiveness of green mould control of a specific application. This is supported by previous work where dip applications gave significantly better green mould control compared to spray application even though spray application loaded significantly higher residue levels (Erasmus et al., 2011 / Chapter 2). Likewise, drench application with the IMZ EC formulation gave superior residue levels (> 2.00 $\mu g.g^{-1}$), but green mould control was poorer when compared to dip treatments of other trials. Smilanick et al. (1997) found that similar IMZ residues loaded through aqueous and wax treatments gave different results in terms of green mould control; the aqueous treatment gave ≈35.0% better control compared to the wax treatment. The benchmark IMZ residue level of 2-3 $\mu g.mL^{-1}$ should therefore be defined in relation to specific application method.

The attempt to model green mould control on IMZ residue levels was compromised by the fact that residue loading using IMZ sulphate was not affected by exposure time, as was anticipated when the study was conceptualised based on earlier findings (Erasmus et al., 2011 / Chapter 2; unpublished results). Nonetheless, the model predicts that a fruit residue of 0.81 $\mu g.g^{-1}$ will control 95% of 4- to 6-hour-old infections of an S isolate with an EC50 of 0.07 $\mu g.mL^{-1}$. Our model further predicts that a fruit residue level of 2.64 $\mu g.g^{-1}$ will curatively control 95% of the 4- to 6-hour-old infections of the R isolate used (EC50 value of 1.83 $\mu g.mL^{-1}$). This concurs with previous findings that infection levels from this R isolate could be lowered from ≈50.0% to ≈7.0% by IMZ residues of ≥ 3.00 $\mu g.g^{-1}$ (Erasmus et al., 2011 / Chapter 2). This places the emphasis on fungicide application, its optimisation, and timely treatment after harvest as the curative action of IMZ diminishes in more established infections (Wild and Spohr, 1989; Smilanick et al., 2006). However, if IMZ resistance development is not monitored and managed diligently, residue levels will continually have to be increased, and a situation will be reached where the MRL will have to be exceeded in order to control IMZ resistant strains of *P. digitatum* (Smilanick et al., 1997).

The very short incubation time of 4-6 h prior to treatment used in this study may not be comparable with reality in industry. Harvested fruit is often kept unmoved for 24 h after harvest before being transported to packhouses to prevent physiological defects, such as oleocellulosis. Drench-treatment in cases where fruit is destined for degreening is generally the first fungicide treatment. In other cases, the first fungicide

treatment is often 2 to 5 days after harvest. In these cases, the ability of IMZ to cure established green mould infections, especially those caused by resistant isolates, will be seriously compromised, if not negated.

Although the focus of this study was not on IMZ resistance, but on residue loading where we showed that dip application can be optimised to give better (increased) residue loading. Caution needs to be taken with the implementation of these methods. Higher residue levels can lead to the selection for stronger resistance levels (Metcalfe et al., 2000; Deising et al., 2008). This will especially be the case in situations where sanitation practices are not up to standard.

Even though the experimental layout in this study did not allow for direct comparisons between fruit types, an interesting observation was that 'Eureka' lemon and Valencia orange fruit appeared to be more resistance to *P. digitatum* infections, especially to the R isolate, than navel orange and Clementine mandarin fruit. 'Valencia Late' oranges were more resistant to *P. digitatum* infection (60.1%) than two blood orange cultivars 'Tarocco' (89.2%) and 'Sanguinello' (76.7%) (D'Aquino et al., 2006). Smilanick et. al. (2008) mentioned that mandarins were more susceptible to infection than lemons and oranges. The seasonal picking window also has an impact on fruit susceptibility to green mould, where early season fruit will be more prone to disease after long storage than late season fruit of the same cultivar (Venditti et al., 2010).

In this study, complete sporulation inhibition was only found on IMZ treated Clementine mandarin fruit infected with the S isolate. The level of sporulation inhibition on IMZ treated fruit of the other citrus fruit kinds never exceeded 50% regardless of isolate. This can be attributed to sub-optimal residue loading of < 2 $\mu g.g^{-1}$. Smilanick et al. (1997) found that residue levels of 2 and 3.5$\mu g.g^{-1}$ were necessary to control sporulation. Smilanick et al. (2006) also showed that aqueous IMZ treatments gave inferior sporulation control when compared to IMZ in wax treatments. Clear indications were, however, observed of reduced levels of sporulation of the S isolate in IMZ treated fruit when compared to the R isolate in this study. This is confirmed by Pérez et al. (2011) who found that sporulation of resistant isolates could not be controlled. The inoculation method in this trial may also not be the most suitable to study IMZ sporulation inhibition. A different method is applied in other work that focused on this aspect (Holmes and Eckert, 1999; Pérez et al., 2011).

South African citrus packhouses have been using the fungicide dip tank with great success despite of the various challenges it offers in terms of sanitation and management. However, sub-optimal residue loading, increasing levels of IMZ resistance and non-satisfactory levels of control indicated that in order to be effective IMZ application must be carefully managed. Results from this work suggest two management options for citrus packhouses that apply IMZ sulphate in dip tanks. Firstly, maintaining the pH level at 6 and restricting exposure time to 45 s, this should load residue levels above 2 µg.g-1. Secondly, maintaining the pH level at 3 and a minimum exposure time of 90 s will result to effective curative control of green mould without the risk of exceeding the MRL. With this second option, residue levels of < 2 $\mu g.g^{-1}$ can be expected, consequently it is advised that an additional IMZ treatment, for example in wax coating application, be applied.

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Table 1. Slopes derived from linear regression lines fitted to residue loading data measured from different citrus types (Clementine mandarin, 'Eureka' lemon, navel and Valencia orange) dipped for 15 to 540 s in a 500 µg.mL^{-1} solution of imazalil (IMZ) sulphate at pH 3 or buffered to pH 6 or 8 with $NaHCO_3$ or NaOH or in a unbuffered 500 µg.mL^{-1} solution of the IMZ EC formulation.

Treatment			Linear slope[a]			
Formulation	**Buffer**	**pH**	**'Clementine' mandarin**	**'Eureka' lemon**	**'Navel' orange**	**'Valencia' orange**
IMZ sulphate	None	3	0.004l	0.005l	0.004l	0.004l
IMZ sulphate	$NaHCO_3$	6	0.035hij	0.032ijk	0.030ijk	0.025jk
IMZ sulphate	NaOH	6	0.040hig	0.031ijk	0.032ijk	0.022k
IMZ sulphate	$NaHCO_3$	8	0.074ab	0.057ef	0.058cdef	0.070bc
IMZ sulphate	NaOH	8	0.061cdef	0.044gh	0.058def	0.062bcde
IMZ EC	None	7	0.084a	0.050fg	0.063bcde	0.069bcd

[a]Each slope followed by the same letter is not significantly different according to Student's t test ($P < 0.05$; LSD = 0.0119)

Table 2. Mean imazalil (IMZ) residue levels measured and green mould infection incidence (%) rated after 21 days at 3.5°C plus an additional 7 – 14 days of incubation at 20°C on Clementine mandarin, 'Eureka' lemon, navel and Valencia orange inoculated with either an imazalil (IMZ) resistant or sensitive isolate of *P. digitatum* and then dipped for various time periods in different concentrations of IMZ sulphate with a solution pH of 3.

Exposure time (s)	Imazalil concentration (µg.mL^{-1})	Residue[a] (µg.g^{-1})	Green mould infection[b]	
			Sensitive	**Resistant**
0	0	0.00	91.0a	79.3b
60	0	0.05a	65.6c	70.8cb
5	250	0.77b	10.7f	66.7c
15	250	0.82b	3.0gf	66.5c
90	250	0.79b	3.9gf	62.4c
15	500	1.53cd	2.4gf	53.2d
90	500	1.36c	3.9gf	49.7d
540	500	1.66d	1.1g	36.0e

[a]Means followed by the same letter do not differ significantly ($P = 0.05$; LSD = 0.223)

[b]Means followed by the same letter do not differ significantly ($P = 0.05$; LSD = 8.85)

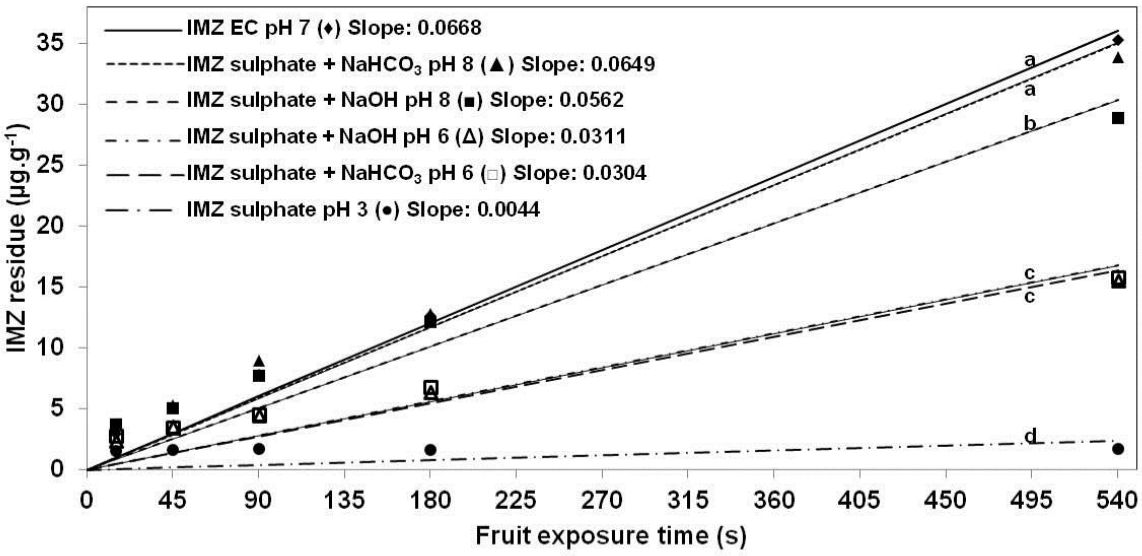

Figure 1. Linear regression lines and mean imazalil (IMZ) residue levels measured on Clementine mandarin, 'Eureka' lemon, navel and Valencia orange fruit that were dipped for 15 to 540 s in a 500 µg.mL^{-1} solution of IMZ sulphate at pH 3 or buffered to pH 6 or 8 with $NaHCO_3$ or NaOH or in a unbuffered 500 µg.mL^{-1} solution of the IMZ EC formulation. Each slope followed by the same letter is not significantly different according to Student's t test ($P \leq 0.05$; LSD = 0.0059) applied after analyses of variance was done.

CHAPTER 4

CURATIVE CONTROL OF CITRUS GREEN MOULD BY IMAZALIL AS INFLUENCED BY INFECTION AGE, WOUND SIZE, FRUIT EXPOSURE TIME, SOLUTION pH AND FRUIT BRUSHING AFTER TREATMENT

Abstract

Green mould (*Penicillium digitatum)* is a major cause of postharvest losses in citrus. Wounds and infection can be induced during the harvest process and should be controlled during postharvest stages to prevent decay. Imazalil (IMZ) in the sulphate formulation is currently applied by the majority of South African packhouses through an aqueous dip treatment. In this study the effects of incubation time (infection age), exposure time, solution pH, wounds size and fruit brushing after dip treatments on residue loading and curative green mould control were investigated. Exposure time did not have a significant effect on residue loading on fruit dipped in pH 3 solutions of IMZ (< 2.00 $\mu g.g^{-1}$). Increasing the pH to 6 resulted in significantly increased residue loading, which increased with longer exposure time, but mostly to levels below the maximum residue level (MRL) of 5 $\mu g.g^{-1}$ after 180 s. Post-dip treatment brushing reduced residue levels obtained in IMZ pH 3 solutions by up to 90% to levels < 0.5 $\mu g.g^{-1}$; however, curative control of the IMZ sensitive (S) isolate was mostly unaffected, but with poor sporulation inhibition. At pH 6, post-dip brushing reduced residues to ≈ 60%; again curative control of the sensitive isolate was unaffected, but with better sporulation inhibition than the pH 3 treatments. Wounded rind sections loaded higher residue levels compared with intact rind sections, and large wounds loaded higher levels than small wounds (≈ 10.19, ≈ 9.06 and ≈ 7.91 $\mu g.g^{-1}$ for large, small and no wound, respectively). Curative control of infections originating from large wounds was significantly better than those from small wounds. The ability of IMZ to control sensitive green mould infections declined from 6 and 12 h after inoculation on Clementine mandarin fruit of infections induced by small and large wounds, respectively; on navel orange fruit, curative control declined 18 and 36 h after inoculation for the respective wound size treatments. This work shows the importance of timeous fungicide treatment after harvest especially on more susceptible citrus types. Results also indicate that excess residues can be stripped from the fruit, retaining residues necessary for curative control in the wound sites. However, reduced residue loading compromised the sporulation inhibition activity of IMZ.

1. Introduction

South Africa is the largest exporter of shipped fresh citrus fruit in the world (Edmonds, 2013). More than 100 million cartons (15 kg) are shipped to countries mostly in the Northern hemisphere. Time from harvest to market can be from 4 weeks up to 8 weeks, which emphasises the need for effective postharvest measures to ensure fruit quality and prevent decay. Green mould caused by *Penicillium digitatum* (Pers.: Fr.) Sacc. is considered to be the main cause of postharvest losses in citrus (Eckert and Eaks, 1989).

Wounds (injury) on the citrus rind are the entry portal for *P. digitatum* to successfully infect (Green, 1932; Nadel-Schiffmann and Littauer, 1956). Deeper injuries (≥ 2 mm) will result in more successful infection and cut-like injuries will be more susceptible than punctures (Smoot and Melvin, 1961). During harvest, injuries are more than likely to be induced and these increase the risk for infection (Rose et al., 1951). When

a wound is induced on the rind of a citrus fruit, volatile compounds are released and if conidia from *P. digitatum* are present in the wound it will be stimulated to germinate by these volatiles (Eckert and Ratnayake, 1994; Droby et al., 2008). The exudations from the injured peel and pectin will in turn stimulate infection and growth of the fungus (Kavanagh and Wood, 1971; Stange et al., 2002). The process from germination to infection can be as short as 4 h at 25°C (Plaza et al., 2003). The current protocol for the majority of growers is to let fruit stand for 24 h after harvest to allow for some wilting and the prevention of oleocellosis. Wild and Spohr (1989) showed that IMZ gave good curative control of infection up to ≈ 30 h after inoculation on Washington navels. This should be investigated on soft citrus which is more susceptible to green mould infections.

Green mould infections initiated during harvest need to be controlled before fruit can be packed. For this imazalil (IMZ) is applied as the sulphate salt formulation by means of an aqueous dip at 500 $\mu g.mL^{-1}$ by the majority (78%) of South African packhouses (Erasmus et al., 2011 / Chapter 2). The majority of other citrus-producing countries apply IMZ as the emulsifiable concentrate (EC) formulation and therefore the bulk of research on IMZ has been using this formulation (Eckert and Kolbezen, 1977; Schirra et al., 1996, 1997; Smilanick et al., 1997, 2005; Cabras et al., 1999; D'Aquino et al., 2006; Dore et al., 2009, 2010).

An IMZ residue level of ≥ 2 $\mu g.g^{-1}$ is regarded to give effective control and sporulation inhibition of green mould (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997). These findings have been mostly made from work done on 24 h curative treatments and with the EC formulation. In previous work done by our group, curative and protective control has been compared in aqueous dip and wax spray applications (Erasmus et al., 2011 / Chapter 2; Njombolwana et al., 2013a). It was shown that the aqueous dip application was better curative than protective. The wax application was better protective, and demonstrated IMZ's ability to inhibit sporulation better than the aqueous dip applications. The ideal residue of 2 – 3 $\mu g.g^{-1}$ was also confirmed by this work. Imazalil sulphate reacts differently at an elevated pH level compared to the EC formulation (Erasmus et al., 2013 / Chapter 3). Exposure time had no effect on residue loading when fruit was dipped in an IMZ sulphate solution at pH 3, but the effect was significant when the pH was elevated to pH 6 or 8 with significantly higher levels of IMZ residue loaded, which increased with longer exposure times. The level of pH 8 is above the pKa level of IMZ (6.5) and was therefore not a recommended option as exposure time should be restricted to prevent exceedence of the MRL (maximum residue level; 5 $\mu g.g^{-1}$) (Erasmus et al., 2011 / Chapter 2).

In the majority of packhouses, aqueous application of IMZ is followed by a set of poly-ethylene roller brushes or sponge rollers to reduce the excess moisture in preparation for the wax application. Preliminary findings from packhouse audit samples have indicated that this treatment reduced IMZ residue loading below the ideal residue level. The effect of this reduction in IMZ residue following dip application on green mould control was unknown. Small wounds take up more IMZ than uninjured rind when applied in water (Brown et al., 1983; Brown, 1984). *Penicillium digitatum* requires wounds for infection, and if wounds take up more IMZ than intact rind it needs to be shown whether reduced residue loading following brushing influences green mould control at the target site (the wound).

The aims of this study therefore were to investigate the effects of delayed curative IMZ treatment after inoculation on green mould control, as well as the effects of exposure time and pH level, wound size and fruit brushing after treatment on IMZ residue loading and green mould control.

2. Materials and methods

2.1 Isolates, fruit preparation, inoculation and treatment

Similar methodology was followed as described previously (Erasmus et al., 2011, 2013 / Chapter 2 &3); these are briefly discussed below with methods unique to this study treated in more detail.

2.1.1. Isolates and spore suspensions

Two isolates of *P. digitatum* were used in all trials unless stated differently, one IMZ sensitive (S) and the other resistant (R). The characterisation and identification of these two isolates were described earlier (Erasmus et al., 2011 / Chapter 2). The isolates were grown on potato dextrose agar (PDA; Difco™ Potato Dextrose Agar, Becon, Dickinson and Company, Sparks, USA) medium in Petri dishes and were re-plated in 2-week cycles. Conidia were harvested from 10- to 14-day-old cultures approximately 1 hour before trials commenced. The surface of a culture was washed with sterile deionised water amended with Tween 20 (Sigma-Aldrich, St Louis, Missouri, USA) at a concentration of ± 0.01 $mL.L^{-1}$. The conidial suspensions were amended to a concentration of 1×10^6 $spores.mL^{-1}$ by means of a haemocytometer. The conidial suspensions were placed on magnetic stirrers to maintain a uniform suspension of spores.

2.1.2. Fruit

Untreated export quality Satsuma (cv. 'Miho wase'), mandarin (cv. 'Nules Clementine'), navel orange (cv. 'Washington') and Valencia sweet orange (cv. 'Midknight') fruit were obtained from various citrus packhouses in the Western Cape province of South Africa during the seasons of 2009 - 2012 as the specific citrus types became available. Fruit were collected directly after harvest and washed with 1 $mL.L^{-1}$ didecyl dimethyl ammonium chloride (Sporekill, ICA International Chemicals, Stellenbosch, South Africa). Before trials commenced, fruit were stored at 3.5 - 7°C for ±3 days. A day before a trial, fruit were transferred from cold storage to ambient (± 20°C) in order for fruit temperature to reach ambient and to allow any condensation to evaporate.

2.1.3. Imazalil treatment

All treatments in this study were dipped in 500 $\mu g.g^{-1}$ IMZ sulphate (Imazacure, 750 $g.kg^{-1}$ SG, ICA International Chemicals, Stellenbosch, South Africa) at ambient temperature (20 – 23°C; unless stated differently).

2.2. The effect of wound size on residue loading

Trials were conducted once on Clementine mandarin, once on navel orange and twice on Valencia orange fruit. Prior to treatment, as many as possible 20-mm diameter circles were drawn along the stylar end, equator and navel end of the fruit, by means of a permanent marker pen. Wounds were made in the centres if these marked circles. Each fruit had the same number of segments with large wounds, small wounds, and no wounds in the different zones on fruit. Fruit were wounded with a triple wound inducer, which consisted of three insect needles placed in a needle clamp to create three small wounds of 0.5 mm wide and 2 mm deep at a triangular distance of 1.5 mm apart; this will be referred to as small wounds. For the large wounds a stainless steel rod with a 2 × 2 mm protruding tip were used. After inducing wounds (< 30 min) a specific IMZ treatment protocol (specified below) that included some or all of the following factors

was followed: exposure time range, different pH levels, different solution temperatures and post-treatment brushing. Fruit were dried after treatment at ambient temperatures and frozen at -20°C until sample preparation was conducted for residue analysis. The rind segments (albedo and flavedo only) in the marked circled sites were dissected from the fruit using a 20-mm round metal test tube cap. Rind sections were pooled per replicate per treatment combination for residue analysis; approximately 24 - 36 segments per treatment were obtained per fruit. Two replicates per treatment were used and the number of fruit per replicate treatment varied from 9 – 12. Residue analysis was done as described below.

2.2.1. Valencia orange fruit (exposure time x pH x temperature)

Fruit were dip treated in an IMZ solution at 20°C and 35°C, pH levels of either 3 or 8 and exposure times of 15, 45, 90, 180 and 540 s.

2.2.2. Clementine (exposure time x pH)

Fruit were dip treated in an IMZ solution at pH levels of either 3 or 6 at exposure times of 1, 15, 45, 90, 180 and 540 s.

2.2.3. Navel (exposure time x pH)

The previous trial (2.2.2.) was repeated on navel oranges but without a 540 s treatment

2.3. Biological efficacy trials

In all trials fruit were treated curatively and inoculated 24 hours before IMZ dip-treatment with either the S or R isolates of *P. digitatum*. Wounding and inoculation were conducted simultaneously by dipping the wound inducer into a spore suspension of *P. digitatum* (1×10^6 spores.mL^{-1}) immediately prior to wounding. Four wounds were induced on each fruit at equal distances around the stem-end; 12 fruit were inoculated per treatment with three replications, unless stated differently. After treatment, fruit from each treatment combination were packed in lock back table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd, Atlantis, South Africa). Each carton was bagged in transparent polyethylene bags. Treated fruit were incubated for 4-5 days at 23°C when infected wounds were rated per fruit by means of a near UV light (UV-A at 365 nm, Labino Mid-light; www.labino.com). Infected wounds could be identified by yellow fluorescence on the surface of the fruit as described by Erasmus et al. (2011; Chapter 2). The three wounds simultaneously induced with the small wound inducer were regarded as one wound in infection ratings. Sporulation was rated 10 – 14 days after inoculation. Infected fruit were given a rating from 1 to 6 related to the fruit area covered with green sporulation: 1 = infection with no sporulation, 2 = sporulation area less than 25% of the fruit, 3 = sporulation area less than 50% of the fruit and more than 25%, 4 = sporulation area more than 50% of fruit and less than 75%, 5 = sporulation area more than 75% of the fruit and less 100%, and 6 = 100% covered with sporulating green mould. Infected fruit were regarded as sporulating when it had a rating of ≥ 4; sporulation incidence was calculated accordingly.

2.3.1. The effect of incubation time on curative green mould control

Fruit were inoculated with the S isolate only by means of large and small wounds. After incubation for 0, 6, 12, 18, 24, 36 and 48 h fruit were dip treated in IMZ for 60 s at a pH level of 3 or 6. The trial was

conducted twice on Clementine mandarin and three times on Navel orange fruit. Within each citrus type trial three replicates with six fruit each were treated. Residue level, number of infected wounds per fruit and number of sporulating fruit were rated.

2.3.2. The effect of exposure time on green mould control

Fruit were inoculated with either the S or R isolate by means of the small wound inducer. Dip treatments commenced in a 500 $\mu g.mL^{-1}$ IMZ solution with a pH level of either 3 or 6 for an exposure time range of 1, 15, 45, 90 and 180 s. This trial was conducted twice on Clementine mandarin, once on navel orange and once on Valencia orange fruit. Four replicates of twelve fruit each were used per treatment. Residue level, number of infected wounds per fruit and number of sporulating fruit were rated.

2.3.3. The effect of fruit brushing after IMZ dip treatment on green mould control

Fruit were inoculated with either the S or R isolate by means of the small wound inducer. After dip treatment in 500 $\mu g.mL^{-1}$ IMZ with exposure times of 1, 15, 45, 90 to 180 s and pH level of 3 or 6, fruit were either brushed over a set of sponge and brush rollers, or not treated further. Directly after dip application the fruit destined for brushing were placed on an elevator which took ± 30 s to transfer the fruit to the brushing unit. The brushing unit consisted of 4 sponge rollers and 6 polyethylene brushes. Fruit residing time on the brush unit varied from 30 – 50 s. The brush unit was saturated with 500 $\mu g.mL^{-1}$ IMZ solution at pH 3 before the first treatment. This was achieved by evenly and manually drenching the rolling brushes with 4 L solution. Following treatment, fruit were left to dry at ambient temperatures. This trial was conducted once each on Satsuma mandarin, Clementine mandarin and Valencia orange fruit. Four replicates of twelve fruit each were used per treatment. Residue level, number of infected wounds per fruit and number of sporulating fruit were rated.

2.3.4. The effect of wound size and post dip-treatment brushing on green mould control

This trial was similar to the one described in 2.3.3., but large and small wounds were also introduced as treatments. Due to the size and logistics of the trial it was only conducted once on Valencia orange fruit. Three replicates of six fruit each were used per treatment. Residue level, number of infected wounds per fruit and number of sporulating fruit were rated.

2.4. Residue analyses

For IMZ residue analyses, six fruit from two separate replicate treatments were sampled from the specific treatments described above, left to dry and frozen (-20°C) until prepared for IMZ residue analysis. The fruit were defrosted, weighed and then macerated to a fine pulp by using a blender (Salton Elite Blender, Almalgamated Appliance Holdings Limited, Reuven, South Africa) and re-frozen. Sub-samples of the macerated fruit were submitted for IMZ (chloramizol) residue analyses by Hearshaw and Kinnes Analytical Laboratory, Cape Town, South Africa. The samples were extracted using acetonitrile followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography tandem mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, California, USA).

2.5. Statistical analyses

Percentage infected wounds per fruit was converted to percentage control in relation to the average infection of fruit in the untreated control. Data were subjected to analyses of variance (ANOVA) and Fisher's test was applied to show significant differences between treatments at a confidence level of 95% using XLSTAT (Addinsoft, www.xlstat.com). In the case of 2.3.1. regression was done on data from the applicable and significant interaction according to the ANOVA. Residue and sporulation incidence data were subjected to ANOVA and Fisher's test.

3. Results

3.1. The effect of wound size on residue loading

3.1.1. Valencia orange fruit (exposure time x pH x temperature)

Analysis of variance for residue data measured on rind segments showed a meaningful pH x temperature x exposure time interaction (P = 0.179; ANOVA not shown). Fruit dipped in a pH 3 solution at either 20°C or 35°C loaded similar residue levels and exposure time did not make a difference (5.71 – 7.37 $\mu g.g^{-1}$; Figure 1). Increasing the pH level to 8 made a significant difference, with almost double the residue after 15-s dip (11.93 $\mu g.g^{-1}$) compared with pH 3 treatments. Exposure time also had a significant effect with a continuous increase in residues after 45, 90, 180 and 540 s (16.17, 28.73, 51.23 and 129.19 $\mu g.g^{-1}$, respectively). Increasing the temperature of the pH 8 solution from 20°C to 35°C resulted in significantly higher residues at 15, 45, 90 and 180 s (23.57, 32.50, 53.04 and 71.47 $\mu g.g^{-1}$, respectively), but similar residues at 540 s (131.13 $\mu g.g^{-1}$) compared with the cooler pH 8 treatments. Wound size was not involved in any significant interactions, but was meaningful as main effect (P = 0.094). Rind segments with large wounds loaded significantly higher IMZ residues (32.54 $\mu g.g^{-1}$), compared with unwounded rind sections (28.34 $\mu g.g^{-1}$), while rind sections with small wounds loaded intermediate levels (30.90 $\mu g.g^{-1}$), which did not differ significantly from large and unwounded sections.

3.1.2. Clementine mandarin fruit (exposure time x pH)

Analyses of variance for residue data from rind segments showed a meaningful wound size x pH x exposure time interaction (P = 0.156; ANOVA not shown). Exposure time made no significant difference in the pH 3 solution, where the mean residue levels loaded over the exposure time range on rind segments with large, small and no wounds were 6.36, 6.03 and 5.06 $\mu g.g^{-1}$, respectively (Figure 2). Residue levels after 1 s exposure time in the pH 6 solution (3.84, 5.58 and 3.81 $\mu g.g^{-1}$ for large, small and no wound segments, respectively) was comparable to those loaded on fruit dipped in the pH 3 solution. Longer exposure time had a significant increasing effect on residue loading in the pH 6 solution. Segments with large wounds loaded significantly higher residue levels at longer exposure times (15.97 to 30.35 $\mu g.g^{-1}$ from 90 – 540 s), while segments with small wounds and no wounds loaded similar levels (10.52 to 22.31 $\mu g.g^{-1}$ and 10.50 to 20.31 $\mu g.g^{-1}$, respectively).

Whole fruit residue levels followed a similar trend for following IMZ pH 3 dips generally and did not differ significantly and ranged from 1.13 – 1.85 $\mu g.g^{-1}$ (Figure 3). However, the residue level following the 1-s dip (1.12 $\mu g.g^{-1}$) increased after 15 and 45 s dips (1.85 and 1.83 $\mu g.g^{-1}$, respectively), thereafter it decreased to 1.51, 1.61 and 1.38 $\mu g.g^{-1}$ after 90, 180 and 540 s, respectively. After 1- and 15-s dips in pH 6 solution,

residue levels (1.71 and 2.12 µg.g^{-1}, respectively) were similar to those following pH 3 dips, but a significant increase was observed for the 45 - 540 s treatments (2.49 – 6.19 µg.g^{-1}).

3.1.3. Navel orange fruit (exposure time x pH)

Analyses of variance for residue loading data measured on navel orange rind segments with different wounds (large, small or none) showed a significant pH x exposure time interaction (P = 0.008; ANOVA not shown) and wound size x pH interaction (P < 0.0001). Corresponding to the Clementine results, the wound size x pH x exposure time results are presented in Figure 4. Residue levels on fruit dipped in the pH 3 solution were similar and ranged from 2.67 – 5.52 µg.g^{-1} regardless of wound treatment. Significantly higher residues increasing with longer exposure times were measured on small and large wounded rind segments at pH 6 and ranged from 5.12 – 10.33 µg.g^{-1}. Residue levels loaded on the pH 6 treated segments with no wounds were similar to those loaded on the small and large wound segments dipped in the pH 3 solution.

Analyses of variance for whole fruit residue data showed that exposure time and pH, separately, was significant as main effects (P = 0.025 and 0.0004, respectively; ANOVA not shown), but results for the interaction are presented in Figure 5. Exposure time had no significant effect on residue loading on fruit dipped in the pH 3 solution which ranged from 1.05 – 1.59 µg.g^{-1}. Increasing the pH level from 3 to 6 led to an increase in residue loading where the levels loaded in the 45 – 180 s treatments were significantly higher (1.90 – 2.25 µg.g^{-1}) than the pH 3 solution.

3.2. Biological efficacy trials

3.2.1. The effect of incubation time on green mould control

3.2.1.1. Residue loading

For both Clementine and navel the residue level was significantly higher on fruit treated in pH 6 (1.95 and 1.75 µg.g^{-1}, respectively) compared to pH 3 (1.25 and 1.15 µg.g^{-1}, respectively).

3.2.1.2. Green mould control

Analyses of variance for percentage green mould control data from Clementine mandarin and navel orange fruit showed a meaningful citrus type x pH x wound size x incubation time interaction (P = 0.069; ANOVA not shown). The effect of pH was only significant in one navel trial where the pH 6 treatments resulted in higher levels of control regardless of incubation time (results not shown) The highly significant citrus type x wound size x incubation time interaction (P = < 0.0001) was further explored by means of regression analyses. Data consisting of means for six replicates in the two Clementine and three navel trials were fitted on the model, $Y = A + (D-A)/(1+(X/C)^B$, to predict the effect of incubation time on green mould control. Table 1 shows the function variables and R^2 values of the prediction models as presented in Figure 6 for Clementine mandarin and Figure 7 for navel orange fruit. On Clementine fruit, curative control started to decline after 12 h incubation on fruit with large wounds, and after 6 h on fruit with small wounds. Treatment after 48 h resulted in a predicted 42.4% and 48.1% control for large and small wounds, respectively. On navel the curative action of IMZ was more effective on older infections compared to Clementine. Curative control

started to decline after 36 h for large wounds and 18 h for small wounds, with a predicted 68.9% and 53.6% control after 48 hours, respectively.

3.2.1.3. Sporulation

No clear trends could be observed for the percentage sporulating fruit (results not shown).

3.2.2. The effect of exposure time on green mould control

3.2.2.1. Residue loading

Analyses of variance for residue level measured on Clementine mandarin and navel and Valencia orange fruit dip treated for an exposure time range (1 – 180 s) in an 500 $\mu g.mL^{-1}$ IMZ solution at either pH 3 or 6 showed a meaningful batch x pH x exposure time interaction (P = 0.054; ANOVA not shown). The reason for this interaction could be firstly ascribed to the first batch of Clementine showing a significant residue level increase with longer exposure time in the pH 3 solution (ranging from1.43 to 2.35 $\mu g.g^{-1}$ for 1 to 180 s; results not shown). This was not evident for the second Clementine batch, on which predictably low residues were loaded without any significant exposure time effect for exposure times of 15 – 180 s (1.51 $\mu g.g^{-1}$). The second reason for the batch interaction was the Valencia batch showing that exposure time had a less of an effect in the pH 6 solution compared to the other fruit batches, with residue levels from 1.23 to 1.94 $\mu g.g^{-1}$ compared with ≈ 1.72 to ≈ 3.12 $\mu g.g^{-1}$ on the other fruit batches for the exposure time range of 1 to 180 s. The batch effect was ignored and the significant pH x exposure time interaction (P = 0.001) interaction is shown in Figure 8. On fruit treated in the pH 3 solution a 1 s dip resulted to the lowest residue level (1.21 $\mu g,g^{-1}$); longer exposure for 15 – 180 s in pH 3 loaded significantly higher residue levels (1.57 – 1.77 $\mu g.g^{-1}$), but these values did not differ significantly. In contrast, fruit dipped for 1, 15, 45, 90 and 180 s in a pH 6 solution loaded significantly higher residue levels and these also differed significantly from each other (1.60, 1.94, 2.24, 2.52 and 2.83 $\mu g.g^{-1}$, respectively). ANOVA also indicated a significant citrus batch x pH interaction (P = 0.002). On each fruit batch, fruit dipped in the pH 3 solutions load significantly lower residue levels compared to fruit dipped in the pH 6 solutions: 1.89 and 2.57 $\mu g.g^{-1}$ and 1.36 and 2.34 $\mu g.g^{-1}$ for the two Clementine batches, 1.70 and 2.36 $\mu g.g^{-1}$and 1.28 and 1.62 $\mu g.g^{-1}$ for navel and Valencia fruit, respectively (Table 2). Valencia fruit that loaded a significantly lower residue level (1.62 $\mu g.g^{-1}$) compared to the other citrus batches at pH 6.

3.2.2.2. Green mould control

Analyses of variance for percentage green mould control data showed a significant citrus batch x isolate x pH interaction (P = 0.006; ANOVA not shown). Green mould infection of the sensitive isolate was significantly better controlled on the second batch of Clementine than the first batch, but at similar levels for pH 3 and pH 6 treatments (72.2% and 71.2% *vs.* 61.5% and 58.3%, respectively; Table 3). The sensitive isolate was significantly better controlled on the navel and Valencia batches than the Clementine batches. For navels, significantly improved control was observed at pH 6 (96.5%) compared with pH 3 (88.6%); however, on Valencia this effect was opposite (94.2 and 86.5% or pH 3 and 6, respectively). The resistant isolate was generally poorly controlled on the Clementine and navel batches (35.0 – 47.8%) regardless of solution pH. These levels were slightly higher on Valencia with pH 6 providing significantly better control than pH 3 (62.8 and 54.0%, respectively).

A significant citrus batch x exposure time x isolate interaction was also shown (P = 0.030). This batch effect was largely ascribed to differences in control of the resistant and sensitive isolate between the different fruit batches as shown above. To portray the effect of exposure time on infection more clearly, the time x isolate interaction (P = 0.226) was described (Figure 9). Mean percentage curative control improved significantly with longer exposure times (1 to 180 s) for the sensitive (72.1 to 85.1%) and resistant isolate (38.6 to 52.5%).

3.2.2.3. Sporulation inhibition

A significant citrus batch x isolate x pH interaction was shown by analysis of variance of the sporulation incidence data obtained from infected fruit (P = 0.043; ANOVA not shown). Sporulation incidence of the sensitive isolate was the lowest on the Clementine batches (< 45.0%; Table 4), with less sporulating fruit in the IMZ at pH 6 treatments (< 33.0%) than on the pH 3 treatments. On the navel and Valencia IMZ at pH 6 treated batches the levels of sporulating infected fruit were generally higher (< 79.0%) than those on Clementine with pH 3 treatments resulting in significantly lower levels (52.6 and 45.7% for navel and Valencia, respectively). For the resistant isolate, significantly higher levels were observed on the Clementine and navel fruit (> 94.0%) regardless of pH level. These levels on Valencia were significantly lower (54.6 and 74.0% for pH 3 and 6, respectively) and similar to those found with the sensitive isolate on Valencia. There was also a significant time x isolate interaction (P < 0.0001), but this was mainly due to the 0 s (untreated) fruit infected with the S isolate having higher levels of sporulating fruit (98.6%; results not shown) compared to treated fruit (32.0 – 45.5%; results not shown). Within the exposure time treatments there were no significant differences. All R isolate infected treatments had similar levels (95.4 – 86.2%), compared with the untreated fruit (93.9%), except for the 180 s treatment (78.8%).

3.2.3. The effect of post dip-treatment brushing on green mould control

3.2.3.1. Residue loading

Analyses of variance for residue data indicated no higher order interaction between fruit types (P = 0.912; ANOVA not shown). The pH x exposure time and the brush treatment x exposure time interactions was significant (P < 0.0001 and = 0.006, respectively). The brush treatment × pH × exposure time interaction (P = 0.914) is shown to demonstrate the effects of these factors (Figure 10). Fruit dipped in the pH 3 solution loaded similar residue levels with very little difference between exposure time treatments (1.03 – 1.69 $\mu g.g^{-1}$). Brushing the pH 3 treated fruit resulted in a significant reduction in residue loading and a similar residue level between exposure times (0.35 – 0.42 $\mu g.g^{-1}$). Significantly higher residue levels were loaded at pH 6 and longer exposure time resulted in a further significant increase (2.16, 2.58, 2.84, 3.26 and 3.62 for 1, 15, 45, 90 and 180 s, respectively). Brush treatment of the pH 6 treated fruit lead to a significant reduction of residue levels (1.31, 1.52, 1.75, 1.89 and 2.27 $\mu.g^{-1}$), but these levels were still significantly higher than the respective pH 3 treatments. The citrus type x pH interaction was also significant (P < 0.0001). The IMZ at pH 6 solution of all citrus types loaded similar residues (2.26 – 2.36 $\mu g.g^{-1}$; results not shown). The residue level loaded in the pH 3 solution was significantly lower and Clementine and Satsuma significantly lower than Valencia (0.57, 0.74 and 1.34 $\mu g.g^{-1}$, respectively).

3.2.3.2. Green mould control

Analyses of variance of percentage control data showed two significant four factor interactions (ANOVA not shown): citrus type x pH x isolate x brush treatment interaction (P = 0.0001) and citrus type x pH x Isolate x time (P = 0.0154). The citrus type interaction was largely ascribed to better control of the sensitive and resistant isolate on Valencia (average of 10% and 39%, respectively) compared to the other fruit kinds (results not shown). To allow a robust interpretation of the treatment effects, results of the brush treatment x pH x isolate x exposure time interaction are presented in Figure 11. Within the majority of all treatments the increase from 1 s to 15 s exposure time resulted in a significant increase in control (from ≈ 78% to ≈ 87% of the sensitive isolate and ≈ 35% to ≈ 48% of the resistant isolate). For the sensitive isolate, increasing the IMZ solution pH from 3 to 6 resulted in a significant increase in control on the majority of 15 – 90 s exposure time treatments (≈ 90 to ≈ 96%). Brushing did not significantly influence the curative control levels obtained at most exposures and for both pH treatments. No significant difference in control of the sensitive isolate was observed after 180 s exposure (≈ 94%) regardless of brushing or pH level. For the resistant isolate, the increasing of pH from 3 to 6 did not significantly influence the level of control for the majority of 15 – 180 s exposure time treatments (≈ 58%). Brushing after IMZ dip-treatment in the pH 3 solution significantly reduced control levels (from ≈ 53 to ≈ 41% control for 15 – 180 s treatments), but not from the pH 6 solution.

3.2.3.3. Sporulation inhibition

Analysis of variance of sporulation incidence data from infected fruit indicated three significant or meaningful three factor interactions: pH x isolate x exposure time (P = 0.001), brush treatment x pH x isolate (P = 0.0019) and citrus type x isolate x exposure time (P = 0.0142). To allow interpretation of sporulation in relation with curative control results, results from the brush treatment x pH x isolate x exposure time interaction (P = 0.1165) are presented in Figure 12. For the sensitive isolate, any length of exposure time in an IMZ solution resulted to a significant reduction of sporulating infections, but pH had a significant effect: an average of 97.2% sporulating fruit at time 0 compared with 64.2 to 38.8% following pH 3 dips, and 30.2 to 23.2% following pH 6 dips (1 – 180 s). Longer exposure times also led to lower sporulation incidences, especially following the pH 3 treatments. Post-dip brushing had a significant effect: no sporulation inhibition was observed on pH 3 dipped fruit (86 – 93%), while sporulation incidence on pH 6 dipped fruit was markedly reduced (49.5 to 36.8%). Sporulation of the resistant isolate could not meaningfully be reduced regardless of treatment; levels ranged from 77.4 to 96.4% compared with the untreated control (average 97.2%). The citrus type x isolate x exposure time interaction (P = 0.0017) showed that exposure time in an IMZ treatment had an effect on citrus types inoculated with the sensitive isolate: 63.8 to 53.6%, 56.0 to 41.1% and 50.7 to 31.4%, respectively for Valencia, Clementine and Satsuma compared to the average control levels at 99.3% (results not shown). The citrus type x exposure time effect was not significant in the case of the resistant isolate.

3.2.4. The effect of wound size and post dip-treatment brushing on green mould control

3.2.4.1. Residue loading

Mean whole fruit residue levels as affected by brushing treatment, pH and exposure time are presented in Figure 13. Valencia fruit that were dipped in the longer exposure times generally loaded

significantly higher residue levels at pH 3 and pH 6. Increasing the pH from 3 to 6 also resulted to a marked increase in residue level (1.35 - 2.01 and 2.08 - 2.58, respectively); significantly at 45 to 180 s exposure times. Brushing fruit after IMZ at pH 3 treatment reduced residue levels loaded in the various exposure time treatments to ≈ 0.17 $\mu g.g^{-1}$, with no difference between exposure times. For the IMZ at pH 6 treatment, brushing reduced residues to 0.51, 0.66, 0.84, 0.98 and 1.05 $\mu g.g^{-1}$ (for 1, 15, 45, 90 and 180 s, respectively).

For wounded rind segments, analyses of variance for residue loading data showed significant interactions for pH x exposure time (P = 0.002; ANOVA not shown), wound size x pH (P = 0.002) and brush treatment x wound size (P = 0.006). Exposure time generally had no effect on residue loading in the pH 3 solution (1.22 – 1.75 $\mu g.g^{-1}$; Table 5). For the IMZ at pH 6 treatment, exposure time had a significant effect and residues increased with longer exposure times (1.96 – 4.11 $\mu g.g^{-1}$ for 1 – 180 s). Segments with large wounds loaded significantly higher residue levels compared to segments with small and no wounds, while segments with large wounds from fruit dipped in an IMZ at pH 6 solution had significantly higher residues than those from the pH 3 solution (4.22 and 2.08 $\mu g.g^{-1}$, respectively; Table 6). Segments with small wounds had significantly lower residue levels following dips in IMZ at pH 3 and 6 (1.49 and 2.67 $\mu g.g^{-1}$, respectively), but significantly higher levels than segments with no wounds (1.02 and 2.18 $\mu g.g^{-1}$, respectively). Brushing after dip treatment significantly reduced residue levels on segments from means of 4.54, 3.24 and 2.49 to 1.76, 0.92 and 0.71 $\mu g.g^{-1}$ on the large, small and no wound segments, respectively (Table 7).

3.2.4.2. Green mould control

Analyses of variance for percentage green mould control data showed a significant brush treatment x isolate x exposure time x wound size interaction (P = 0.002; ANOVA not shown). Control of the sensitive isolate on fruit dipped for 1 to 180 s and left unbrushed varied between 92.6 and 100% for large wounds and 89.3 to 100% for small wounds (Figure 14) with longer exposure times leading to improved control. Brushing did not lead to a significant reduction in curative control on fruit with large or small wounds (95.7 to 100% control), except for a significant reduction in control on 1-s dip plus brushed fruit with small wounds (79.6%). The resistant isolate was generally significantly better controlled if it was inoculated with a large wound (69 to 91%) compared with small wounds (41 to 71%), with longer exposure times also leading to significantly better control. Brushing resulted in a significant reduction in the control level for large and small wounds (56 to 75% and 35 to 66%, respectively), with the exposure time effect still evident.

The factors brush treatment x pH x isolate x wound size also showed a significant interaction (P = 0.001). No significant difference in control of the S isolate was observed between IMZ at pH 3 and pH 6 treatments (94.3 to 99.6%; Table 8). However, the R isolate was significantly better controlled in large wounds and at pH 6 treatments, while brushing generally led to significant reductions in control.

3.2.4.3. Sporulation inhibition

Due to many treatments inoculated with the sensitive isolate showing 100% control, the dataset for sporulation incidence had too many missing values. Hence, data were analysed ignoring exposure time as factor, thereby comparing treated fruit (times 1 to 180 s) with untreated fruit (time 0). Analyses of variance showed significant interactions for brush treatment x pH x wound (P = 0.008; ANOVA not shown) and pH x isolate (P < 0.0001). The brush treatment x pH x isolate x wound were discussed to highlight differences between the sensitive and resistant isolate (P = 0.232). IMZ treated and unbrushed fruit inoculated with the

resistant isolate tended to have higher levels of sporulation (39.7 – 90.8%) compared to the sensitive isolate (0.0 – 66.7; Table 9). Fruit inoculated with the sensitive isolate by means of large wounds (24.1 – 25.0%) and small wounds at pH 6 (0.0%) had significantly lower sporulation levels than sensitive small wound infections at pH 3 (66.7%); the latter treatment combination also had significantly lower sporulation levels when inoculated with the resistant isolate (39.7%) than the large and small wound-pH 3 treatments (69.6 – 90.8%). Brushing increased sporulation incidence levels on IMZ treated fruit inoculated with the sensitive isolate with small wound-pH 3 treatments yielding significantly more sporulating fruit (90.0%) than the other treatments (41.7 – 52.5%). For the resistant infections on brushed fruit, no significant sporulation inhibition was evident relative to the control fruit.

4. Discussion

Various important outcomes were highlighted in this study: the curative control of green mould by IMZ as demonstrated by previous studies was confirmed, but our study more specifically investigated factors such as incubation time, pH, exposure time, wound size as well as the effects of post-dip brushing treatments (which is a standard packhouse treatment, but to our knowledge not considered in previous green mould control studies) and the effects thereof on curative control and sporulation inhibition.

The exposure time trials confirmed previous work that residue loading was generally not affected by longer exposure time in a pH 3 solution of IMZ sulphate (Erasmus et al., 2013 / Chapter 3). Some indication was, however, observed that there might be fruit batch effects where some moderate increase in residue loading was evident after increased exposure time. The reason for this is not clear and should be investigated further. Nonetheless, residue levels following IMZ at pH 3 dips rarely exceeded 2 $\mu g.g^{-1}$ even after 3 min exposure. This study also confirmed the effect of dip treatment in higher pH IMZ solutions (Erasmus et al., 2013 / Chapter 3), which led to significant increases in residue level. At elevated pH levels, residue loading increased with longer exposure times. This relates to previous reports using the EC formulation. Smilanick et al. (1997) showed that increasing the exposure time from 15 to 60 nearly doubled the IMZ residue loaded in a 37.8°C IMZ EC solution from 2.88 to 5.35 $\mu g.g^{-1}$. Cabras et al. (1999) and Erasmus et al. (2013; Chapter 3) showed similar effects, but with different temperatures and concentrations. In the present study, the MRL of 5 $\mu g.g^{-1}$ was never exceeded in any dip treatment in a pH 6 IMZ sulphate solution except on Clementine after long exposure times. This is contrary to results found in a previous study, where the MRL was exceeded after 161 s at pH 6 (Erasmus et al., 2013 / Chapter 3). The most relevant difference between the two studies was solution temperature. Erasmus et al. (2011; Chapter 2) found that solution temperature of 35°C made no difference compared with 20°C in residue loading from a pH 3 and pH 8 solution of IMZ sulphate. All treatments in the study by Erasmus et al. (2013; Chapter 3) were conducted with solution temperature of 35°C and the current study was conducted using ambient solution temperatures. Although similar trends were still evident there is an indication that temperature may have an effect on residue loading, especially in a pH 6 IMZ sulphate solution. Residue loading from the imazalil EC formulation was affected by temperature (Smilanick et al., 1997; Cabras et al., 1999). However, the effect of temperature on residue loading of the IMZ sulphate formulation should be investigated further.

Wound size played an important role in residue loading and subsequent green mould control. Although not always significant, rind segments with large wounds generally had higher residue levels than

segments with smaller wounds, and rind segments with small wounds tended to load higher residue levels compared to unwounded rind segments. This was also found by others (Brown et al., 1983; Brown, 1984). It should be noted that the small wound treatments in our study consisted of three small wounds. Hence, the difference between large and small wounds would even be larger if we used just one needle instead of three. Control of infections initiated from large wounds was also better than those from small wounds, which is most probably attributed to the higher residue level loaded in large wound sites. Dore et al. (2010) showed that wounded albedo took up more IMZ residues following aqueous dip applications with the IMZ EC formulation and this was increased with the addiction of 2% sodium bicarbonate. This increase was up to 6-fold with or without the addition of 2% sodium bicarbonate. According to them the exposed albedo in the wound site may be the reason for increased residue loading measured from the specific segments.

The pKa value of IMZ is ≈ 6.5 (Siegel et al., 1977; Erasmus et al., 2011 / Chapter 2). Below this level the molecule is more protonated and less lipophilic. This could explain why higher residue levels were loaded at pH 6 than at pH 3. Brown et al. (1990) have shown that the majority (77.5%) of IMZ residues will still be in the exocarp of the peel 7 days after a 15 s dip treatment in 1000 $\mu g.mL^{-1}$ IMZ EC. This is the outer part of the peel containing the epicuticular wax and cuticle. The rest of the residue migrated to the mesocarp. At higher pH the IMZ sulphate formulation will be more lipophilic and should reside in the exocarp where there are more fatty or oily compounds for the IMZ to bind with. In an injury the mesocarp is exposed and can take up more IMZ. Brown et al. (1983) also found that 94% of the IMZ residue was found in the exocarp. They showed that aqueous applied IMZ moved better into the injured part of a rind than when it was applied with wax. This explain in part why in our study we found higher residue loading on rinds injured with the large wound compared to small and no wounds. More parenchyma cells were exposed with the large wound compared to the small wounds, whereby the IMZ solution penetrated wounds more readily and loaded higher residue levels. This part of the rind is also the preferred part that *P. digitatum* will first attack (Barmore and Brown, 1980; Soule and Grierson, 1986). Through staining it was shown that larger wounds (97%; 1 mm diameter) took up more aqueous IMZ solution than smaller wounds (75%; 0.3 mm diameter) (Brown, 1984). Infections originating from these large wounds were also better controlled (> 40% better) than the small wounds. IMZ applied as aqueous dip treatment is therefore a very good targeted application as residue loading in the wound site, *i.e.* potential infection site, is significantly better than on unwounded citrus rinds.

As mentioned earlier, IMZ residue loading differed between citrus fruit types, as well as between batches of the same type and cultivar. In a trial involving two Clementine batches IMZ residue levels of 1.43 – 2.35 $\mu g.g^{-1}$ and 0.72 – 1.54 $\mu g.g^{-1}$ were loaded for exposure time 1 to 180 s in a pH 3 solution on the two separate batches. However, green mould control was significantly poorer on fruit from the first batch despite the higher residue levels. The difference in control could be ascribed to fruit quality or maturity differences. Smilanick et al. (2005) showed that the natural pH level in 2 mm wounds decreased with lemon fruit age and this made the conditions more susceptible for green mould infection. However, the difference in residue loading between fruit batches of the same cultivar is not well understood. Schirra et al. (2000) found no difference in IMZ residue loading on 'Star Ruby' grapefruit harvest at different maturity stages. This variation in residue levels from similar treatments with similar concentrations was also found by Brown at el. (1983) and regarded as "disconcerting", but they cited other reports with similar findings, indicating that this is not an uncommon phenomenon.

The study of IMZ residue loading in unwounded and wounded rind segments yielded some interesting results. As was experienced with whole fruit residue results, IMZ dip treatment at higher pH levels resulted in increased residue loading. However, on rind segments the effect was more dramatic. Increasing the pH level from 3 to 8 resulted in significant increases on wounded Valencia rind segments of up to 97%. pH level increased from 3 to 6 reduced this percentage increase to 10 – 46% on Clementine and 32 – 64% on navel rind segments. Longer exposure time resulted in higher residue loading on wounded segments. Likewise, the effect of brushing following dip treatments was significant: the reduction in potential residue loading on wounded peel discs was up to 87% on fruit dipped in a pH 3 solution and 64% in fruit dipped in a pH 6 solution. Brown and Dezman (1990) treated fruit for 15 s by means of a non-recovery spray applicator, with or without IMZ saturated brushing afterwards, and showed that the brushing did not reduce the IMZ residue significantly. Their study was with the IMZ EC formulation and brushes were continually saturated. Brush treatment in our study was designed to simulate industry practice where the aim is to reduce excess moisture from the fruit surface.

The majority of previous studies on green mould control by IMZ were focused on its curative action on a specific infection age, mostly 24 h (Brown et al., 1983; Brown, 1984; Smilanick et al., 1997). These studies exclusively used the IMZ EC formulation, but did indicate excellent curative action of IMZ. Kanetis et al. (2007) compared IMZ with other fungicides (pyrimethanil, fludioxonil and azoxystrobin) in terms of curative ability and showed that it outperformed fludioxonil and azoxistrobin while comparing well with pyrimethanil. The maximum incubation time tested was 21 h. Our study used the IMZ sulphate formulation and demonstrated the difference in curative action between citrus types as well as infections originating from different wound sizes as affected by infection age and pH of the solution. On Clementine soft citrus fruit, IMZ could effectively cure 12-h old green mould infections originating from large wounds before levels of control started to decline. For small wounds, curative control started to decline after 6 h incubation. On navel orange fruit, curative control started to decline after 18 h for small wound infections and 36 h for large wound infections. The difference between soft citrus and orange fruit can be ascribed to inherent difference in susceptibility to *P. digitatum* infection, and confirms previous findings (Smilanick et al., 2008; Erasmus et al., 2013 / Chapter 3). These findings were similar to those of Wild and Spohr (1989) who showed that infections on fruit treated at 20°C with IMZ (500 $\mu g.mL^{-1}$) could still be cured when treatment was delayed by 30 h. The wounds they used, *i.e.* 2 mm deep with a nail, could be described as large wounds relative to those from our study. Infections caused by the resistant isolate were relatively poorly controlled, but always better if infections originated from large wounds. Differences in curative control of small and large wound infections could be ascribed to significantly higher IMZ residue loading in the large wounds as demonstrated in this study and discussed above. Larger wounds should be easier to remove from fruit destined for packing through the sorting and inspection process. However, smaller wounds might escape the sorting process, load lower residue levels and infections might survive the curative IMZ treatment. This can in part be addressed by increasing the exposure time in an aqueous IMZ dip treatment. Longer exposure time generally led to better curative control of the sensitive isolate, regardless of pH and residue level.

It is common for harvested citrus fruit to remain untreated by a fungicide for longer than 24 h after harvest, as packers often allow some moisture loss or wilting in order to reduce the risk for rind disorders such as oleocellosis. *Pencillium* inoculum is always present in orchards and wounds are bound to be induced during the harvest process (Rose et al., 1951). Fungicide application within 12 to 24 hours after harvest is therefore essential for curative control of infections originating from the harvest processes. The

majority of early season fruit needs to be degreened. Many citrus packhouses in South Africa and USA treat fruit in harvest bins by means of drenching before it is placed in degreening rooms. Smilanick et al. (2006) have shown that this treatment can be very effective with thiabendazole. In a previous study we have shown that through drench with IMZ EC formulation a residue of > 2 $\mu g.g^{-1}$ could be loaded with fairly good control (Erasmus et al., 2011 / Chapter 2). However, compared with dip application a spray-on application was inferior in terms of green mould control, despite higher residues loaded with following spray application (Erasmus et al., 2011 / Chapter 2). Smoot et al. (1971) showed that washing and treating fruit with fungicide before degreening resulted in lower levels of decay. Other work confirmed that there is a limited time between inoculation and treatment for fungicides to give effective curative control (Seberry, 1969; Wild and Spohr, 1989). Orihuel-Iranzo (1991) related delays (10 – 14 days) between harvest and fungicide treatment to postharvest losses at the end of the value chain. In order to address the need for timeous fungicide application after harvest, the efficacy of IMZ and its alternatives following drench applications needs to be investigated further. Additionally, the typical delay between drench treatment and subsequent in-line packhouse treatments, as well as the favourable conditions for green mould development in degreening rooms, should exacerbate fungicide resistance development in *Penicillium* populations. Strategies to curb fungicide resistance should focus on inoculum reduction through sanitation and the use of alternative chemistry or fungicide mixtures (Bancroft et al., 1984; Taverner and Cunningham, 1997).

The IMZ resistant isolate was poorly controlled regardless of increased residues acquired through longer exposure times and higher pH levels. The majority of these control levels were < 50%. The only exception was on Valencia orange fruit. Control levels of up to 90% of R isolate infections could be reached with residue levels of 1.28 – 2.36 $\mu g.g^{-1}$. Here is again evidence of some inherent resistance that Valencia orange fruit have against green mould (D'Aquino et al., 2006; Erasmus et al., 2013 / Chapter 3). In a previous study it was shown that resistant isolates of *P. digitatum* was less competitive than sensitive isolates, but not necessarily less virulent (Holmes and Eckert, 1995). Our work indicates that the specific resistant isolate used had a tendency to have lower infection levels on untreated Valencia orange fruit compared to the sensitive isolate. This was also found in a previous study (Erasmus et al., 2013 / Chapter 3) and needs to be investigated further.

Wax application on citrus fruit is an important aspect necessary to reduce water loss and ultimately preserve quality (Njombolwana et al., 2013a, 2013b). Due to aqueous treatments commonly used for fruit washing and fungicide application, fruit need to be thoroughly dried before wax application. For this air blowers on roller brushes and sponges are implemented in the majority of packhouses. By removing the excess fungicide solution on the fruit, fungicide actives are also removed. This study showed that on fruit dipped in a pH 3 solution of IMZ sulphate; a mean of 75%, but up to 92%, of a potential residue can be removed by post-dip brushing. At pH 6, residue stripping by post-dip brushing was on average 44% (up to 64%). However, curative control of the sensitive isolate could still be achieved, and reduced control was only observed following short exposure times of fruit with small wound infections. Satsuma was the most affected by the reduction in residue that resulted in lower control levels; however, the loss in control might be attributed to Satsuma being more sensitive to green mould than the other citrus types and that the 24-h old infections challenged in that trial might have been too old for effective curative control.

The trials conducted in this study were not designed specifically to study IMZ sporulation inhibition. High levels of control of the sensitive isolate often led to too few infected fruit from which sporulation could be rated. Previous studies focusing on this aspect were conducted by injecting the spore suspension into

the core of treated fruit (Brown et al., 1983; Brown and Dezman, 1990). Nonetheless, our results again confirmed IMZ's ability to inhibit sporulation of sensitive *P. digitatum* isolates, and that sporulation of the resistant isolate could not be inhibited (Eckert et al., 1994; Erasmus et al., 2011; 2013 / Chapter 2 & 3; Pérez et al., 2011; Njombolwana et al., 2013a, 2013b). Higher residue levels generally resulted in lower incidences of sporulating fruit, but total inhibition of sporulation was never achieved from any of these aqueous treatments. The brushing trials further illustrated the effect of residue level on sporulation incidence: although curative control was not affected, the reduced whole fruit residue resulted in poor sporulation inhibition on infected fruit. Our results support those of Smilanick et al. (1997) that an IMZ residue of 2 – 3.5 $\mu g.g^{-1}$ is ideal if sporulation inhibition.

Almost all experimental trials on fungicides are conducted by means of a dip application where fruit are left to dry post treatment. Our study has shown that a standard industry practice such as brushing after dip treatment can have a substantial effect on residue loading and disease control. Despite the fact that IMZ residues were substantially reduced by post-treatment brushing, the effect thereof on curative green mould control of the sensitive PD isolate was negligible. The latter was ascribed to targeted residue loading in wound sites, which also highlighted that the advised 2 – 3 $\mu g.g^{-1}$ (measured as whole fruit residue analysis) was not required for effective curative control of sensitive isolates. However, at these reduced residue levels sporulation was poorly inhibited. The effect of reduced IMZ residues on protective PD control is also not known. For protective control a second IMZ application could be considered with the wax application (Smilanick et al., 1997), which should provide good protective control and sporulation inhibition (Njombolwana et al., 2013a, 2013b). We have shown that the delay between inoculation and treatment should be much shorter (12 to 24 h) for soft citrus compared to oranges for curative action of IMZ to be effective. Timeous postharvest fungicide application within 24 h of harvest is therefore of the utmost importance. However, in order to prevent fungicide resistance development, the use of other green mould fungicides with different modes of action to IMZ should be investigated, as should be the optimising of their application by means of conventional or novel application systems.

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Table 1. Function variables and R^2 values for data consisting of means of green mould control data from six replicates in two Clementine mandarin and three navel orange trials fitted to the model Y = A + (D-A)/(1+(X/C)^B to predict the effect of incubation time of 0 to 48 h on green mould control on fruit dipped in 500 µg.g^{-1} IMZ solution after it were wound inoculated with an IMZ sensitive isolate of *Penicillium digitatum* in a large or small wound.

Citrus type	Wound size	Function variables and standard deviations				R^2
		A	B	C	D	
Clementine	Large	20.6 (31.8)	3.3 (1.4)	37.1 (11.1)	93.7 (2.6)	0.68
	Small	45.4 (6.3)	3.4 (1.3)	21.1 (2.5)	93.9 (2.9)	0.66
Navel	Large	-3591.9 (48434270.8)	10.9 (367.1)	74.5 (91616.6)	99.6 (1.1)	0.53
	Small	-2523.6 (165555.2)	3.9 (2.6)	133.1 (2253.0)	99.5 (1.3)	0.71

Table 2. . Mean imazalil residue level (µg.g^{-1}) measured on two batches of Clementine mandarin and one batch each on navel and Valencia orange fruit that were dip treated in 500 µg.mL^{-1} IMZ at pH 3 or 6 at 20ºC.

Citrus type	Imazalil residue (µg.mL^{-1})[a]	
	pH 3	pH 6
Clementine 1	1.89 c	2.57 a
Clementine 2	1.36 e	2.34 b
Navel	1.70 cd	2.36 ab
Valencia	1.28 e	1.62 d

[a]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 3. Mean percentage curative green mould control on Clementine mandarin, navel and Valencia orange fruit that were inoculated with an IMZ sensitive or resistant isolate of *P. digitatum* 24 h prior to dip treatment in a 500 µg.mL^{-1} IMZ solution at 22°C with a pH of 3 or 6.

Citrus kind	Green mould control (%)[a]			
	Sensitive isolate		Resistant isolate	
	pH 3	pH 6	pH 3	pH 6
Clementine 1	61.5 e	58.3 ef	40.0 ijk	35.0 k
Clementine 2	72.2 d	71.2 d	44.1 hi	36.0 jk
Navel	88.6 bc	96.5 a	42.4 hij	47.8 gh
Valencia	94.2 ab	86.5 c	54.0 fg	62.8 e

[a]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 4. Mean percentage green mould sporulation incidence on Clementine mandarin, navel and Valencia orange fruit that were inoculated with an IMZ sensitive or resistant isolate of *P. digitatum* 24 h prior to dip treatment in a 500 µg.mL^{-1} IMZ solution at 22°C with a pH of 3 or 6.

Citrus batch	Sporulating infected fruit (%)[a]			
	Sensitive isolate		Resistant isolate	
	pH 3	pH 6	pH 3	pH 6
Clementine 1	35.3 b	18.0 a	94.4 ef	96.7 f
Clementine 2	44.7 bc	32.5 ab	98.6 f	99.0 f
Navel	52.6 c	75.0 d	97.9 f	98.7 f
Valencia	45.7 bc	78.7 de	54.6 c	74.0 d

[a] Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 5. Mean imazalil residue levels (µg.g^{-1}) measured on wounded and unwounded rind segments removed from Valencia orange fruit dip treatment in 500 µg.mL^{-1} imazalil at either pH 3 or 6 at exposure times of 1 to 180 s.

Exposure time (s)	Imazalil residue (µg.g-1)[a]	
	pH 3	pH 6
1	1.22 a	1.96 b
15	1.40 a	2.56 c
45	1.55 ab	3.04 cd
90	1.75 ab	3.46 d
180	1.75 ab	4.11 e

[a]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 6. Mean imazalil residue levels (µg.g^{-1}) measured on wounded (large or small) and unwounded rind segments removed from Valencia orange fruit dip treated in 500 µg.mL^{-1} imazalil at either pH 3 or 6 at exposure times of 1 to 180 s.

Wound size	Imazalil residue (µg.g^{-1})[a]	
	pH 3	pH 6
Large	2.08 c	4.22 e
Small	1.49 b	2.67 d
None	1.02 a	2.18 c

[a]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 7. Mean imazalil residue levels (µg.g^{-1}) measured on wounded (large or small) and unwounded rind segments removed from Valencia orange fruit brushed or left unbrushed after dip treatment in 500 µg.mL^{-1} imazalil at either pH 3 or 6 at exposure times of 1 to 180 s.

Wound size	Imazalil residue loading (µg.g^{-1})[a]	
	Dip	Dip plus brushing
Large	4.54 a	1.76 d
Small	3.24 b	0.92 e
None	2.49 c	0.71 e

[a]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 8. Mean percentage green mould control on Valencia orange fruit that were wound inoculated with a large or small wound and an IMZ sensitive or resistant isolate of *Penicillium digitatum* 24 h before dip treatment in 500 µg.mL^{-1} imazalil at pH 3 or 8 and brushed or left unbrushed.

Solution pH	Green mould control (%)[a]			
	Dip		Dip plus brushing	
	Large wound	Small wound	Large wound	Small wound
Sensitive isolate				
3	96.6 a	94.0 a	97.4 a	94.4 a
6	99.5 a	99.6 a	97.6 a	94.3 a
Resistant isolate				
3	78.7 c	66.5 de	67.5 d	35.1 g
6	86.1 b	60.1 ef	72.3 cd	54.4 f

[a] Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 9. Mean percentage green mould sporulation incidence on Valencia orange fruit that were inoculated with a large or small wound and an IMZ sensitive or resistant isolate of *P. digitatum* 24 h prior to dip treatment in 500 µg.mL^{-1} imazalil at pH 3 or 6, after which fruit were brushed or left unbrushed.

Isolate	Solution pH	Sporulation incidence (%)[a]			
		Dip		Dip plus brushing	
		Large wound	Small wound	Large wound	Small wound
Sensitive	Control	100.0 a	100.0 a	100.0 a	100.0 a
	3	24.1 de	66.7 abcd	52.4 bcd	90.0 a
	6	25.0 de	0.0 e	52.5 bcd	41.7 cde
Resistant	Control	66.7 abcd	66.7 abcd	66.7 abcd	66.7 abcd
	3	90.8 a	69.6 abc	54.3 bcd	76.0 ab
	6	78.9 ab	39.7 de	84.0 a	84.7 a

[a] Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

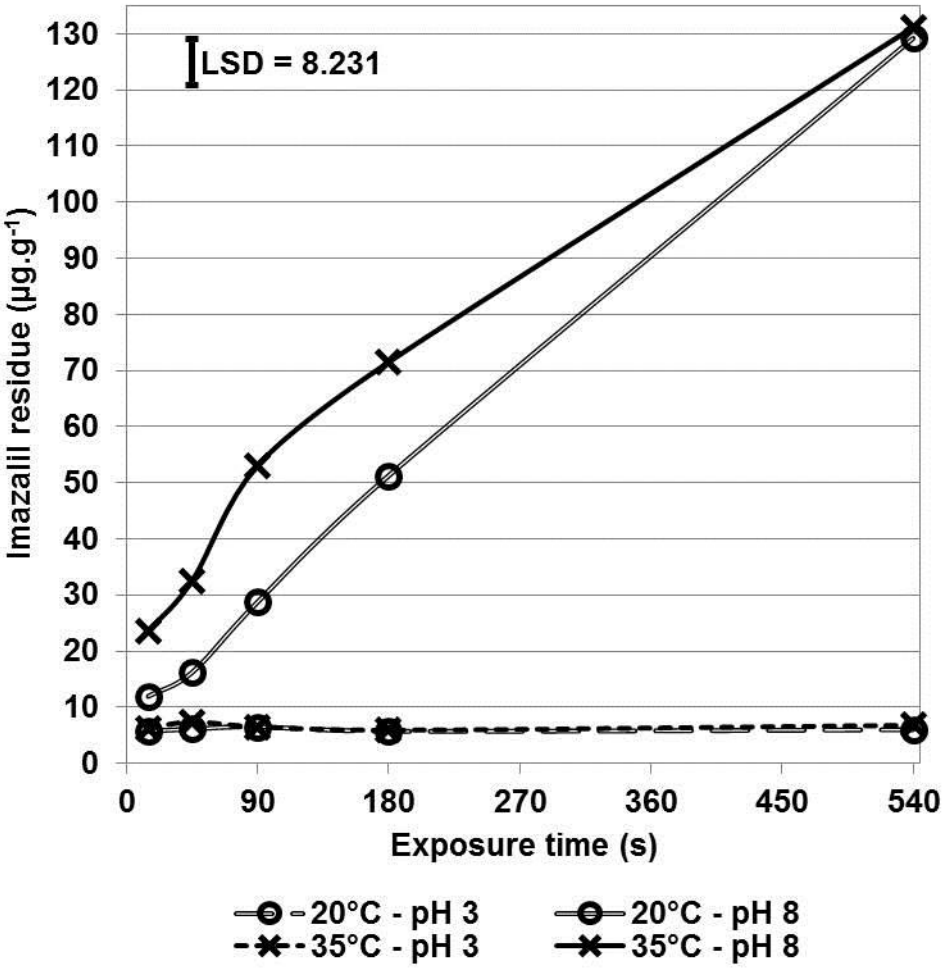

Figure 1. Mean imazalil residue level (µg.g^{-1}) measured on Valencia orange rind segments removed from fruit that were dip-treated for exposure times ranging from 15 – 540 s in a 500 µg.mL^{-1} IMZ solution with a pH level of 3 or 8 and temperature 20 or 35°C. The LSD value (8.231) was determined by means of Fisher's test.

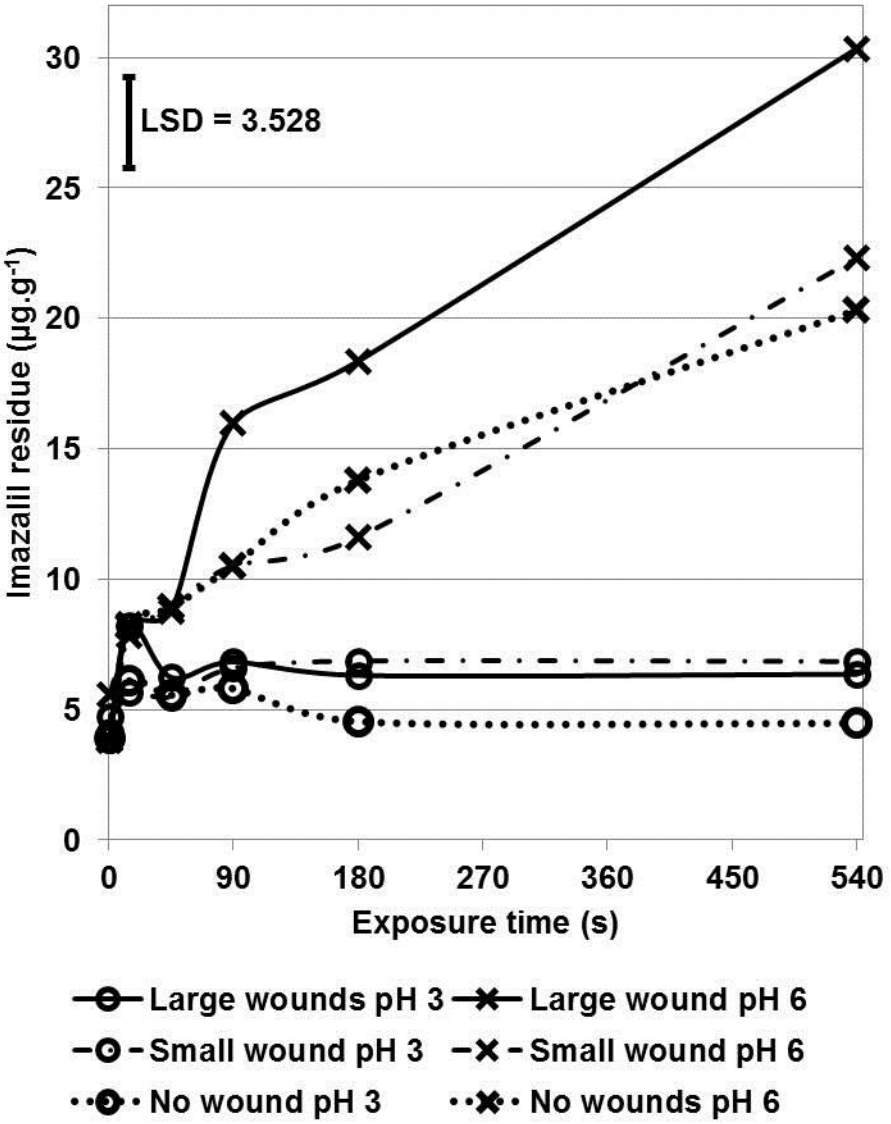

Figure 2. Mean imazalil residue level (µg.g^{-1}) measured on Clementine rind segments with a large, small or no wounds removed from fruit that were dip treated in 500 µg.mL^{-1} IMZ with a pH level of 3 or 6 at an exposure time ranging from 1 – 540 s. The LSD value (3.528) was determined by means of Fisher's test.

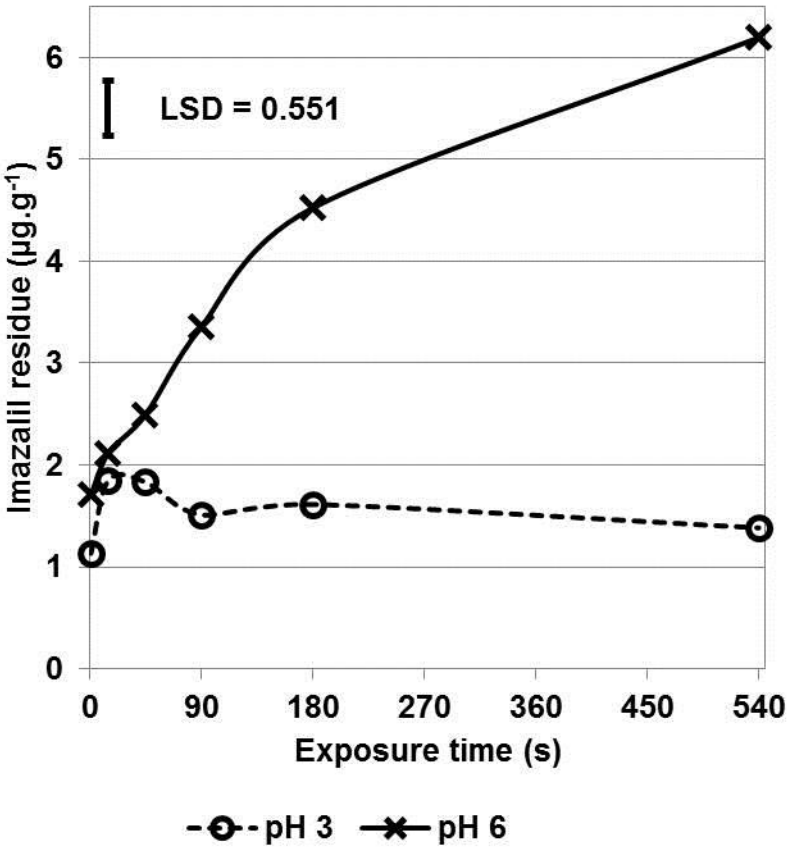

Figure 3. Mean imazalil residue level (µg.g^{-1}) measured on Clementine mandarin fruit that were dip treated in 500 µg.mL^{-1} IMZ at pH 3 or 6 at 20°C with an exposure time range of 1 – 540 s. The LSD value (0.551) was determined by means of Fisher's test.

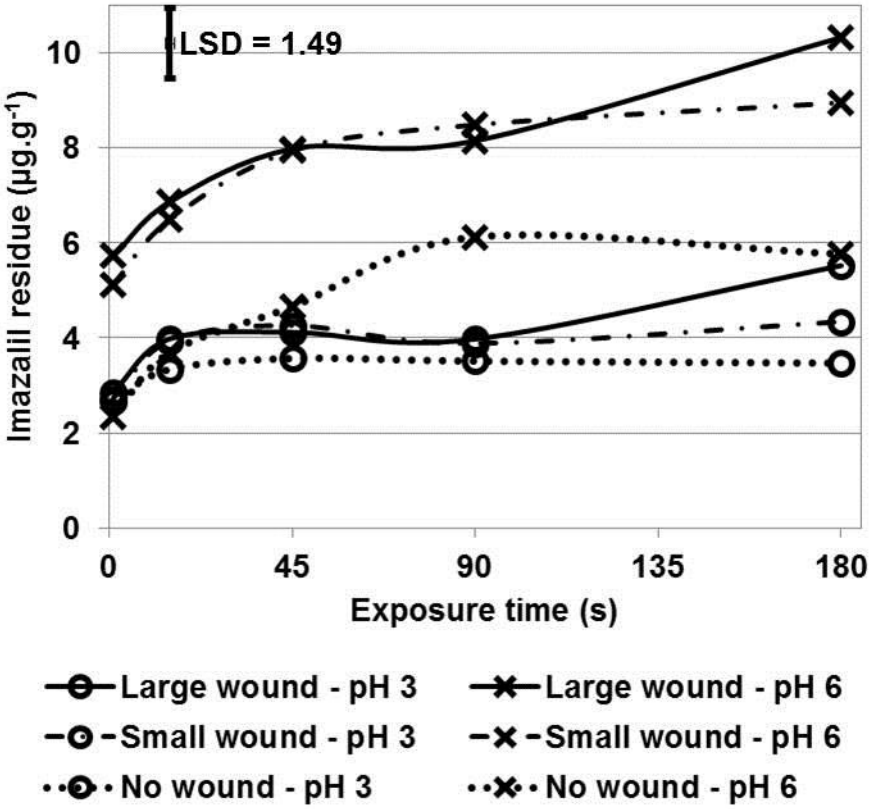

Figure 4. Mean imazalil residue level (µg.g^{-1}) measured on navel orange rind segments with a large, small or no wounds that were removed from fruit dip treated for exposure times of 1 – 180 s in 500 µg.mL^{-1} IMZ with a solution pH of 3 or 6. The LSD value (1.490) was determined by means of Fisher's test.

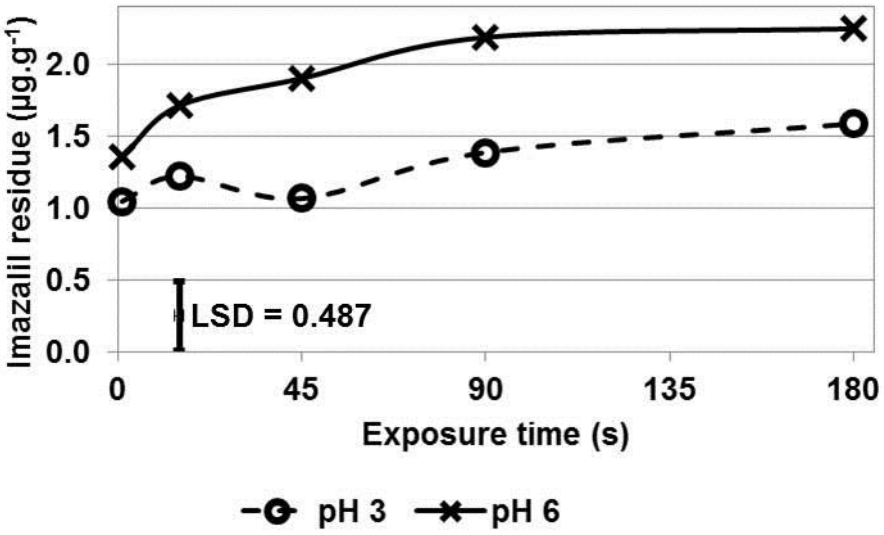

Figure 5. Mean imazalil residue level (µg.g^{-1}) measured on navel orange fruit that were dip treated in 500 µg.mL^{-1} IMZ at pH 3 or 6 at 20ºC with exposure times of 1 – 180 s. The LSD value (0.487) was determined by means of Fisher's test.

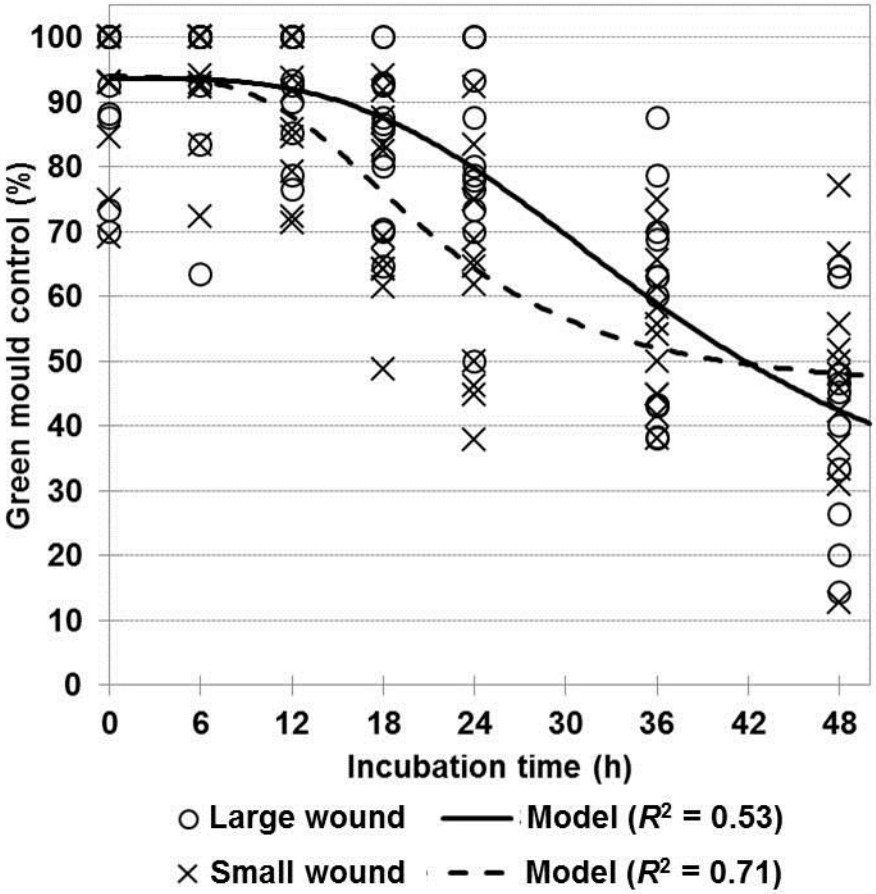

Figure 6. Predicted and measured green mould control (mean %) on Clementine mandarin fruit inoculated with an IMZ sensitive isolate of *Penicillium digitatum* in a large or small wound and incubated for 0 to 48 h prior to a 60 s dip treatment in 500 µg.mL^{-1} IMZ solution at 22°C. Data were fitted on the model, Y = A + (D-A)/(1+(X/C)^{B}, using mean values of six replicates from two trials.

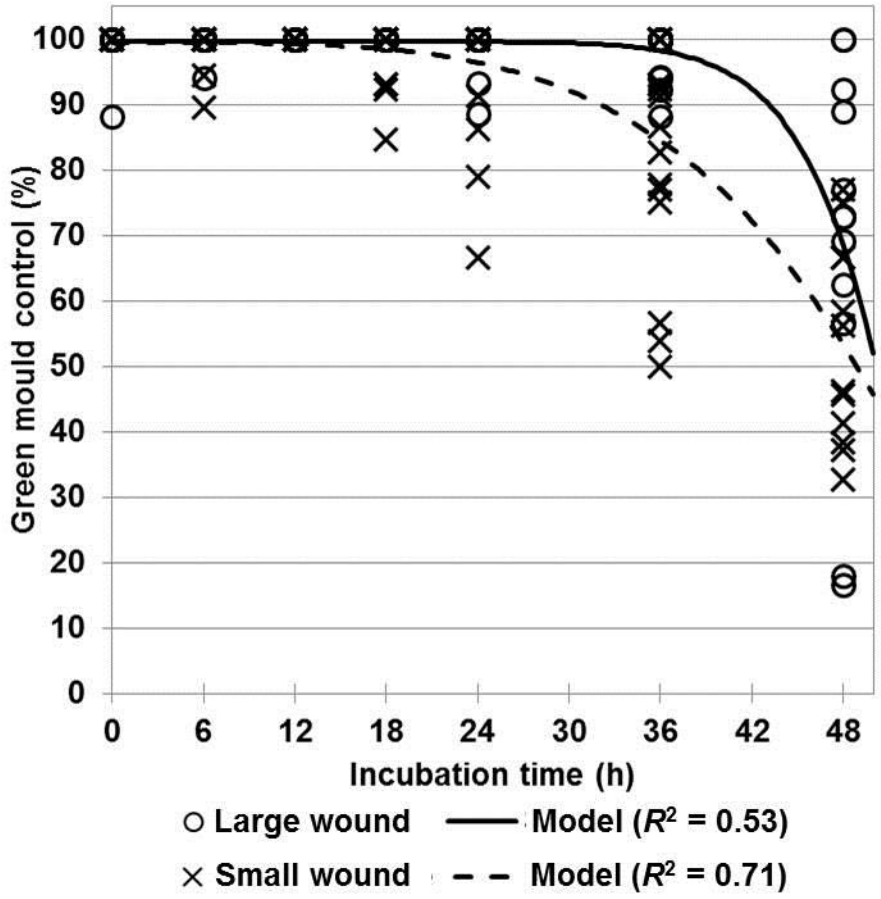

Figure 7. Predicted and measured green mould control (mean %) on navel orange fruit inoculated with an IMZ sensitive isolate of *Penicillium digitatum* in a large or small wound and incubated for 0 to 48 h prior to a 60 s dip treatment in 500 µg.mL^{-1} IMZ solution at 22°C. Data were fitted on the model, Y = A + (D-A)/(1+(X/C)^{B}, using mean values of six replicates from two trials.

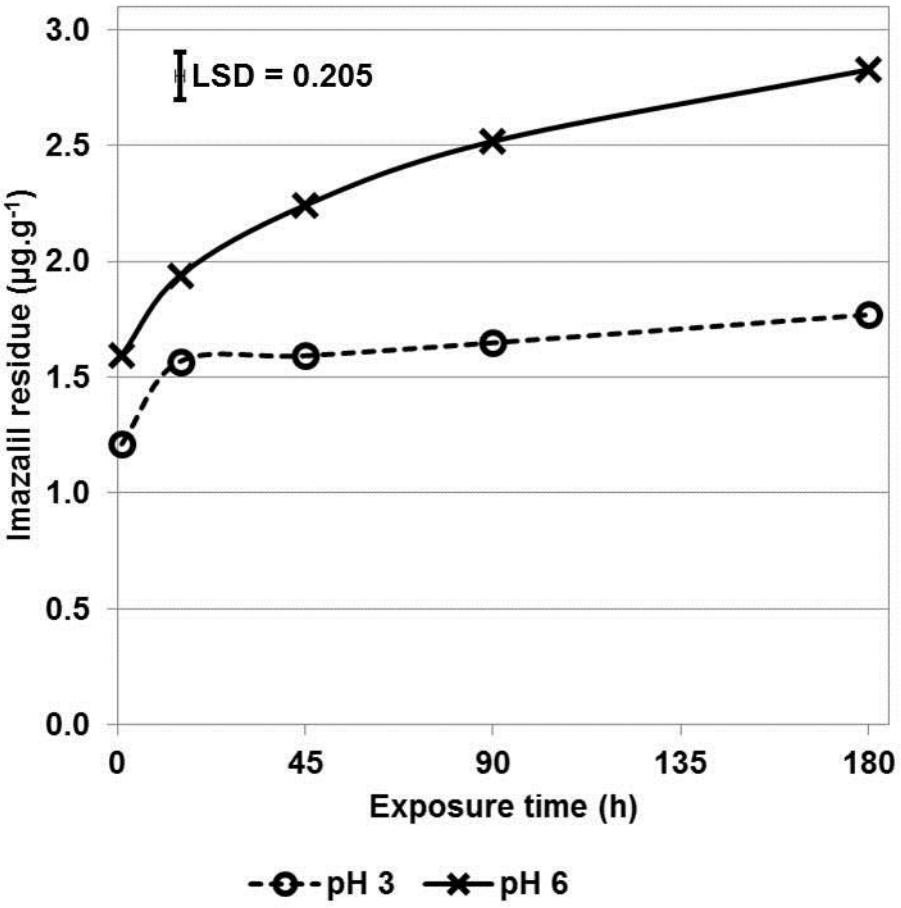

Figure 8. Mean imazalil residue level (µg.g^{-1}) measured on citrus fruit that were dip treated in 500 µg.mL^{-1} IMZ at pH 3 or 6 at 20ºC with exposure times of 1 – 180 s. The LSD value (0.205) was determined by means of Fisher's test.

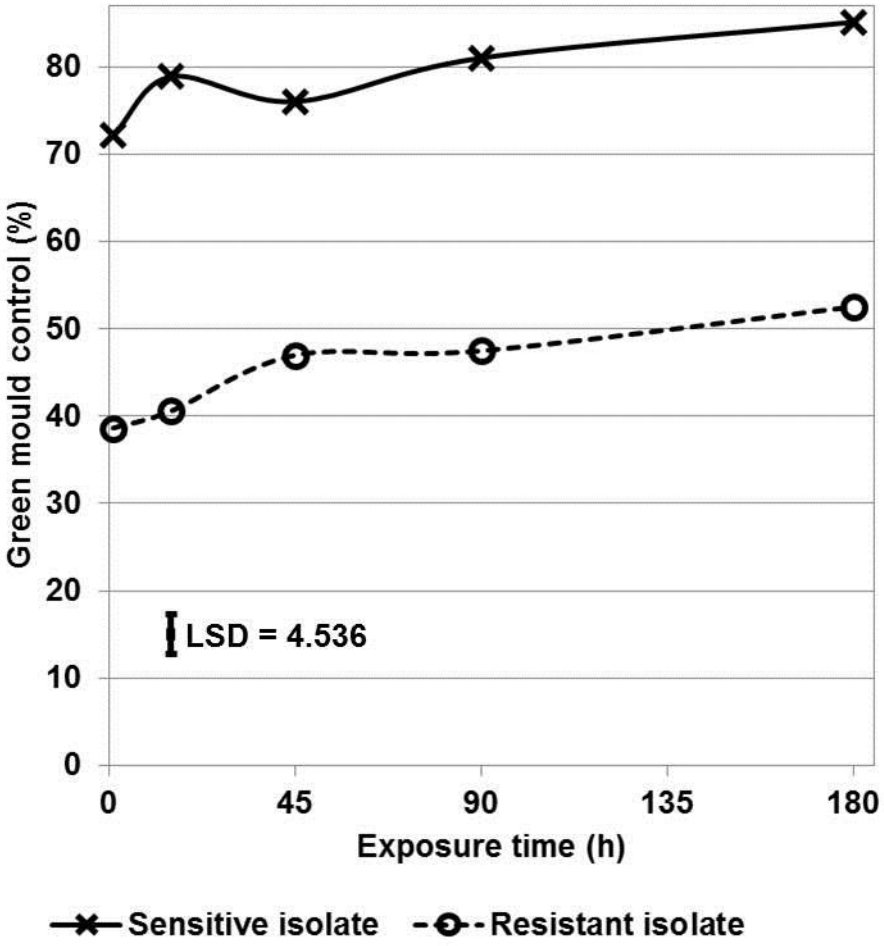

Figure 9. Mean percentage curative green mould control on citrus fruit that were inoculated with an IMZ sensitive or resistant isolate of *P. digitatum* 24 h prior to dip treatment in a 500 µg.mL^{-1} IMZ solution at ≈ 22°C. The LSD value (4.536) was determined by means of Fisher's test.

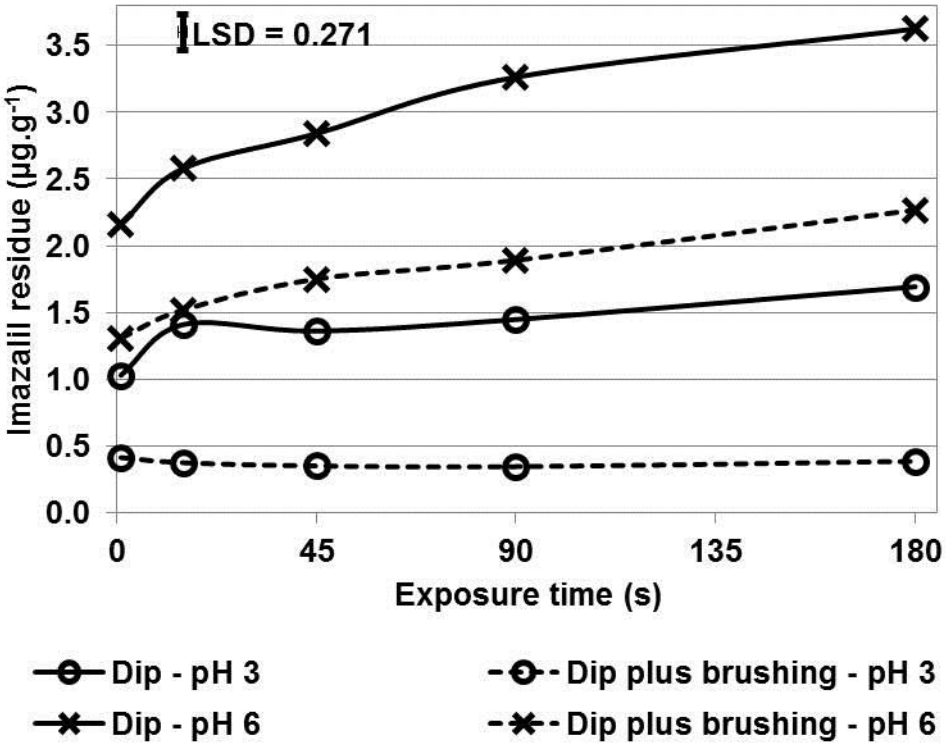

Figure 10. Mean imazalil residue levels measured on Clementine and Satsuma mandarin and Valencia orange fruit that were brushed or left unbrushed after dip treatment with 500 µg.mL^{-1} imazalil at pH 3 or 6 at exposure time from 1 – 180 s. The LSD value (0.271) was determined by means of Fisher's test.

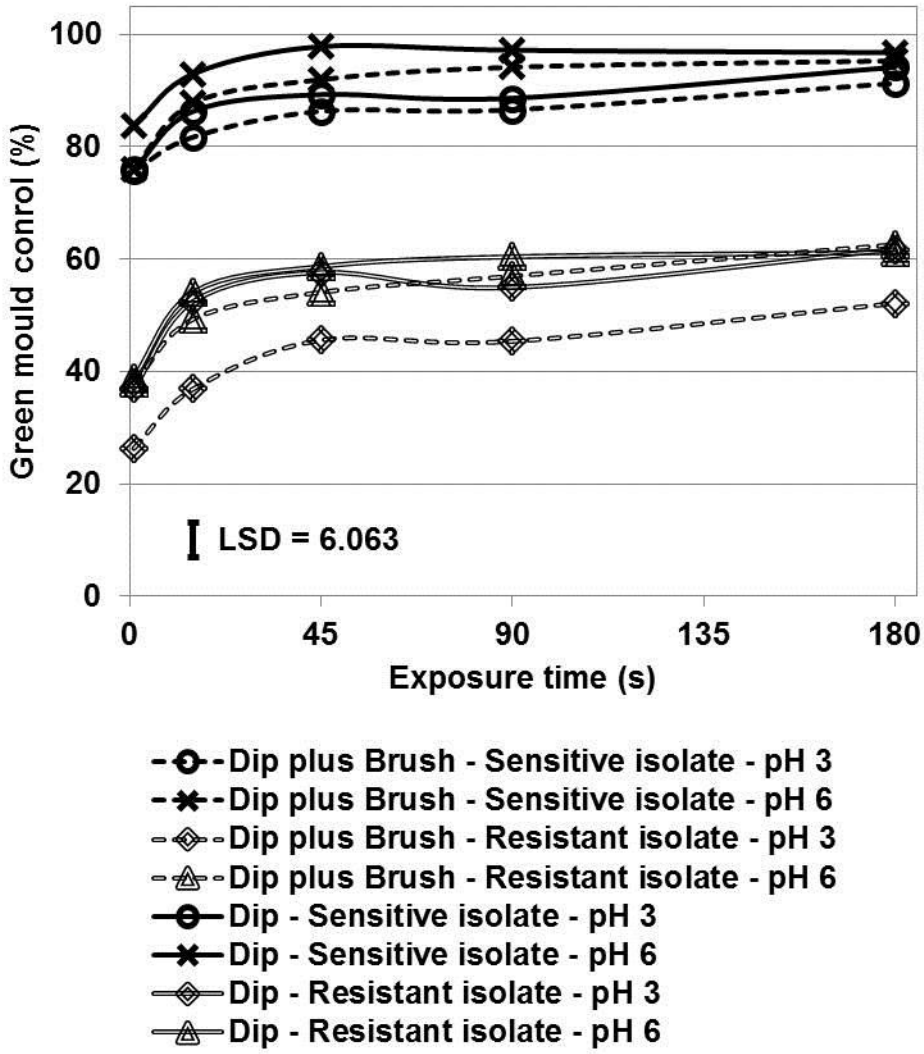

Figure 11. Mean percentage curative green mould control on Clementine and Satsuma mandarin and Valencia orange fruit that were inoculated with an IMZ sensitive or resistant isolate of *P. digitatum* 24 h prior to dip treatment in 500 µg.mL^{-1} imazalil at pH 3 or 6 at exposure time from 1 – 180 s, after which fruit were brushed or left unbrushed. The LSD value (6.063) was determined by means of Fisher's test.

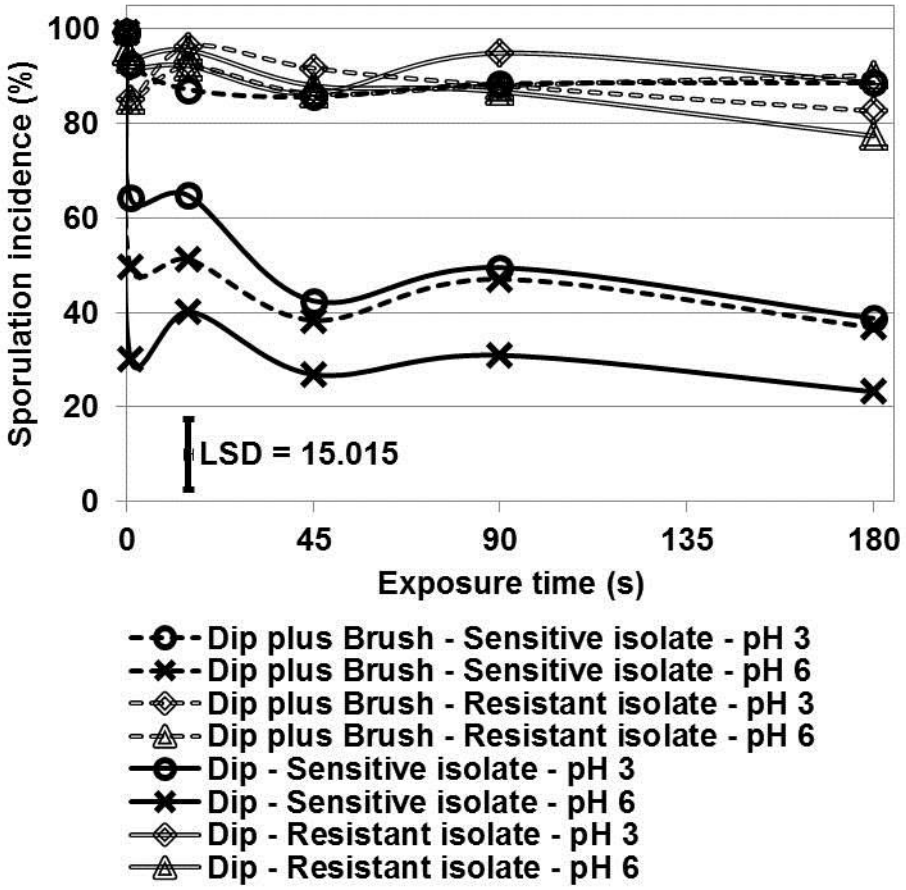

Figure 12. Mean percentage green mould sporulation incidence on Clementine and Satsuma mandarin and Valencia orange fruit that were inoculated with an IMZ sensitive or resistant isolate of *P. digitatum* 24 h prior to dip treatment in 500 µg.mL^{-1} imazalil at pH 3 or 6 at exposure time from 1 – 180 s, after which fruit were brushed or left unbrushed. The LSD value (15.015) was determined by means of Fisher's test.

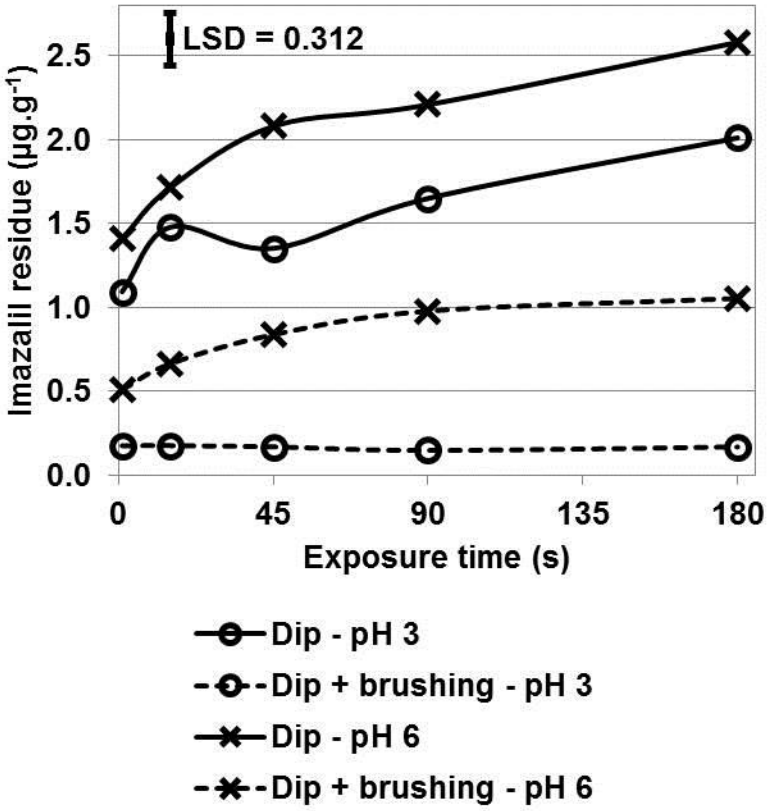

Figure 13. Mean imazalil residue levels measured on Valencia orange fruit that were brushed or left unbrushed after dip treatment in 500 µg.mL^{-1} imazalil at pH 3 or 6 at exposure time from 1 – 180 s. The LSD value (0.312) was determined by means of Fisher's test.

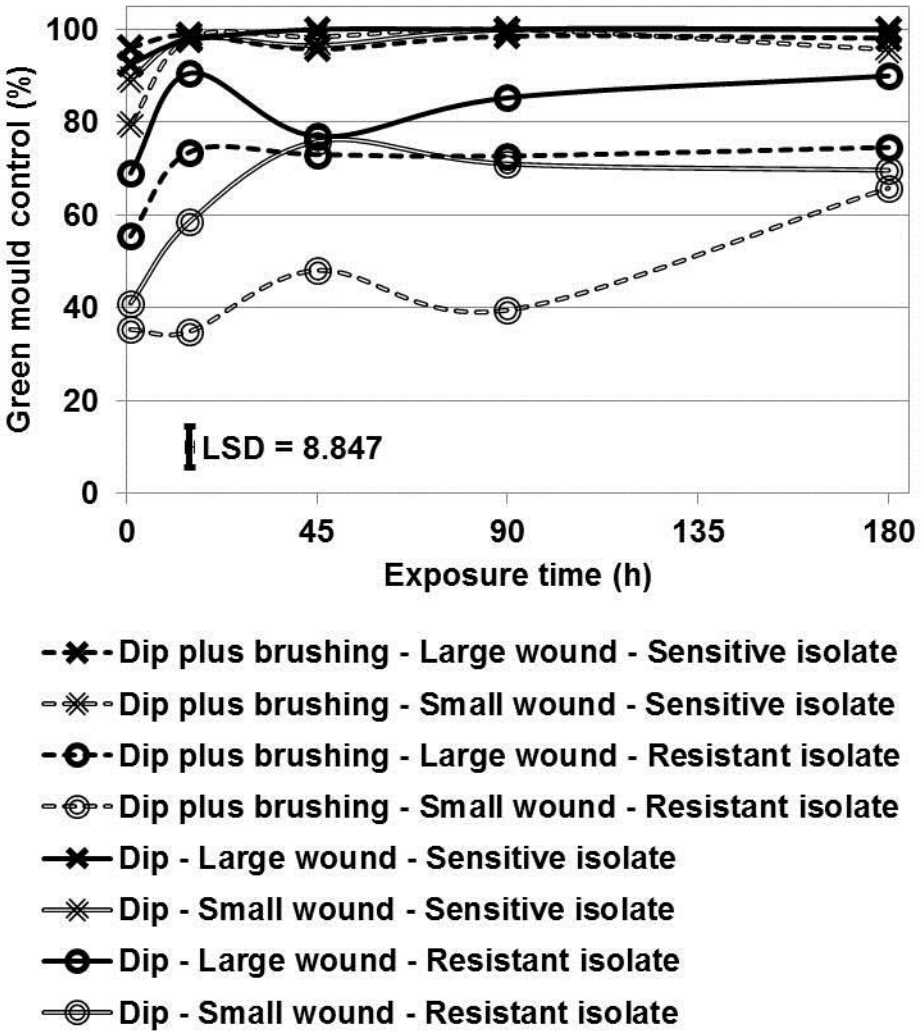

Figure 14. Mean percentage green mould control on Valencia orange fruit that were wound inoculated with a large or small wound and an IMZ sensitive or resistant isolate of *Penicillium digitatum* 24 h before dip treatment in 500 µg.mL^{-1} imazalil for exposure times of 1 to 180 s and brushed or left unbrushed. The LSD value (8.847) was determined by means of Fisher's test.

CHAPTER 5

PRACTICAL IMPACT OF IMAZALIL RESISTANCE ON CONTROL OF POSTHARVEST CITRUS GREEN AND BLUE MOULD

Abstract

Citrus green and blue mould, caused by *Penicillium digitatum* (PD) and *P. italicum* (PI), respectively, are mostly controlled by means of postharvest fungicide applications. Currently, IMZ is regarded as the most effective fungicide in use. Effective IMZ concentrations that inhibit 50% (EC_{50}) growth of nine PD and five PI isolates were assessed *in vitro* and the various isolates categorized according to their resistance (R) factors. Effective residue levels that provided 50% curative ($ER_{50}C$) and protective ($ER_{50}P$) control of these isolates were determined *in vivo*. All the PI isolates were sensitive, having EC_{50} values of 0.005 - 0.050 $\mu g.mL^{-1}$. Three PD isolates were sensitive (0.027 – 0.038 $\mu g.mL^{-1}$), while one resistant isolate was categorized as low resistant (R-factor of 19), one as moderately resistant (R-factor of 33.2), three as resistant (R-factor of 50 - 57.6) and one as highly resistant (R-factor of 70.7). Sensitive PD isolates had mean $ER_{50}C$ and $ER_{50}P$ values on Valencia orange fruit of 0.29 and 0.20 $\mu g.g^{-1}$, and 0.33 and 0.32 $\mu g.g^{-1}$ on navel fruit, respectively. ER_{50} values for resistant isolates did not always correlate with EC_{50} values and ranged from 1.22 – 4.56 $\mu g.g^{-1}$ for $ER_{50}C$ and 1.00 – 6.62 $\mu g.g^{-1}$ for $ER_{50}P$ values. $ER_{50}P$ values for resistant isolates could not be obtained on navel orange fruit, but $ER_{50}C$ values (1.42 – 1.65 $\mu g.g^{-1}$) were similar to those obtained on Valencia fruit. The PI isolates all behaved similar to the sensitive PD isolates with $ER_{50}C$ and $ER_{50}P$ values on navel and Valencia fruit < 0.38 $\mu g.g^{-1}$. Alternative fungicides were assessed for the control of an IMZ sensitive, resistant and highly resistant PD isolate; these included sodium *ortho*-phenylpenate (SOPP), thiabendazole (TBZ), guazatine (GZT), imazalil (IMZ), pyrimethanil (PYR) and Philabuster® (PLB; a combination of IMZ and PYR), fludioxonil (FLU), azoxystrobin (AZO), Graduate®A^+ (a combination of FLU and AZO) and propiconazole (PPZ). Multiple fungicide resistance was shown to IMZ, GZT, TBZ and PPZ in both resistant isolates. For the sensitive isolates, IMZ, SOPP, TBZ, GZT and PLB provided best curative control, while IMZ, GZT and PLB provided best protective control. For the IMZ-resistant isolates, SOPP, PYR and PLB gave the best curative control, while none of the fungicides provided adequate protective control.

1. Introduction

South Africa is the largest exporter of shipped fresh citrus fruit worldwide (Edmonds, 2013). Due to harvest and postharvest handling processes fruit can incur injuries and these make the fruit susceptible to green and blue mould (Rose et al., 1951). Green mould is caused by the pathogen *Penicillium digitatum* (Pers.:Fr.) Sacc (PD) (Smith, 1897; Eckert and Eaks, 1989). It is regarded as one of the major causes of losses due to decay on South African export fruit (Pelser, 1977). *Penicillium italicum* Wehmer (PI), which causes blue mould,

is often neglected in literature due to the main focus mostly being on PD. *Penicillium italicum* is of economic importance due to being more adapted for growth and development at lower temperatures (< 4°C) than PD (Wyatt and Parish, 1995; Plaza et al., 2003) Shipping and storage temperature protocols require temperatures <10°C for the majority of exported citrus cultivars. Both these pathogens require a fresh wound as shallow as 0.25 mm for successful infection (Kavanagh and Wood, 1971). It takes as short as 4 h from germination to establishment of infection (Plaza et al., 2003) and a water-soaked lesion should be visible around the infection site 3 days after infection. Seven to 10 days after inoculation the whole fruit rind could be covered with sporulating green or blue mould (Smilanick et al., 2006). Except for the losses due to the actual decayed fruit, the sporulating infections also soil the neighbouring fruit and this has a further economic impact.

Due to long-distance export routes, the South African citrus industry has to rely on fungicides for the control of postharvest diseases. Currently there are six registered postharvest fungicides for the control of green mould in the South African citrus industry. Prochloraz, which is not favoured commercially, due to apparent reduced efficacy (Keith Lesar, pers. comm.). Sodium ortho-phenyl-phenol (SOPP) has been in use for > 50 years (Johnson et al., 2001) but is not commonly used in South Africa, mostly due to the possible risk of phytotoxicity if not managed well. Resistance to SOPP has already been reported in the early 1960s (Harding, 1962). Thiabendazole is widely used in South Africa and world-wide, especially in the drench and wax applications. It has been in use since the late 1960s, soon after which resistance development in green and blue mould populations were reported (Harding, 1972). Guazatine (GZT) is the only green mould fungicide that also has good curative action against sour rot (*Geotricum citri-aurantii*), but is only allowed in member countries of Codex Alimentarius (www.codexalimentarius.org). GZT has been in use since the early 1980s (Brown, 1988). Wild (1983) reported GZT resistance already in 1983. Imazalil (IMZ) is the most effective and reliable green mould fungicide currently in use. Laville (1977) reported first on IMZ's efficacy in the late 1970s and registered use started in the early 1980s (Bus et al., 1991); soon after the case of IMZ resistance was reported (Eckert, 1987). Pyrimethanil (PYR) has more recently been introduced as green mould fungicide. It became available for citrus use in California more than two decades after the registration of IMZ (Kanetis et al., 2007; Kanetis et al., 2008). The combination product, Philabuster® (PLB, which contains IMZ and PYR; Janssen PMP, Belgium) was developed and registered for use on citrus against green mould. Resistance to PYR has already been reported in field populations, but not yet from the packhouse environment (Kinay et al., 2007; Kanetis et al., 2008). Other active ingredients being evaluated for postharvest citrus use are fludioxonil (FLU) and azoxystrobin (AZO), and were found to have some activity against green mould (Kanetis et al., 2007; Kanetis et al., 2008). These researchers also showed that the combination of the two actives (FLU and AZO) had potential. Graduate®A^{+} [GRA); Syngenta, USA] has been developed and may have some potential for the control of green mould. Fludioxonil is still new and being registered in certain countries (D'Aquino et al., 2013); no resistance have been reported yet to our knowledge. Finally, propiconazole (PPZ) was reported to have an action against green mould (McKay et al., 2012). The fungicides FLU, AZO, GRA and PPZ are not registered in South Africa for postharvest use on fresh citrus fruit.

The majority (> 78%) of South African packhouses apply IMZ in a sulphate formulation by means of a fungicide dip tank (Erasmus et al., 2011 / Chapter 2). This application gives variable results in terms of residue loading due to differences in exposure time, solution pH and concentration. A survey conducted by Erasmus et

al. (2011; Chapter 2) indicated that the median residue level loaded was 1.02 µg.g^{-1} with the lowest level at 0.24 µg.g^{-1} and the highest level at 3.85 µg.g^{-1}. The ideal IMZ residue level for control and sporulation inhibition is regarded as 2 µg.g^{-1} (Kaplan and Dave, 1979; Brown et al., 1983; Brown and Dezman, 1990; Smilanick et al., 1997; Erasmus et al., 2011 / Chapter 2). The maximum residue level (MRL) is 5 µg.g^{-1}, but certain markets demand even lower levels (Cranney, 2012). In previous studies, an IMZ residue level of ≈ 1 µg.mg^{-1} was shown to give adequate control of an IMZ sensitive (S) isolate of PD and ≥ 2 µg.mg^{-1} was needed to control 80 - 95% infections caused by an IMZ resistant (R) isolate (Erasmus et al., 2011, 2013 / Chapter 2 & 3). However, the previous studies were conducted using one S and one R isolate only and with 4 – 6 h incubation time. To our knowledge information on effective IMZ residue levels for control of PI is not documented.

The mechanism of IMZ resistance development is described as the over-expression of the gene P*d*CYP51, which will increase the amount of P405-dependant sterol 14α-demethylase, which will in its turn affect IMZ-sensitivity (Hamamoto et al., 2000b; Ghosoph et al., 2007; Kiralj and Ferreira, 2008). Resistance to IMZ is polygenic and involves 21 genes on 8 loci and is linked with 6 groups; hence, it is theoretically more difficult for *Penicillium* to develop resistance to this fungicide (Laville et al., 1977). IMZ-resistant isolates of *P. digitatum* are generally less fit on fruit not treated with IMZ when compared to IMZ-sensitive isolates (Dave, Sales & Walia 1989; Holmes, Eckert 1995, Kinay et al. 2007). This loss of fitness was only evident when the IMZ-resistant isolate is in competition with the IMZ-sensitive isolate and is not well understood (Holmes, Eckert 1995).

In this study, a number of PD and PI isolates with various levels of sensitivity to IMZ were used to determine effective IMZ residue levels for the curative and protective control of both *Penicillium* species following IMZ application in fungicide dip tanks. Additionally, this study determined the efficacy of 9 active ingredients or combinations thereof as alternatives for IMZ, specifically against resistant isolates to understand their potential role in an anti-resistance fungicide program.

2. Materials and methods

2.1. Penicillium *isolates and inoculum preparation*

Nine PD and five PI isolates were used in trials done for this project. Seven PD and all five PI isolates were obtained from University Pretoria (UP; South Africa). The sensitive and resistant PD isolates used in previous work (Erasmus et al., 2011, 2013 / Chapter 2 & 3) were included in the study and were coded PD3 and PD5, respectively. The UP isolates were coded PD1, PD2, PD4, PD6, PD7, PD8 and PD9. The five PI isolates were coded PI1, PI2, PI3, PI4 and PI7.

In order to obtain inoculum for biological efficacy tests, the isolates were grown at ambient temperature on potato dextrose agar medium (PDA; Difco™ Potato dextrose agar, Becton, Dickinson and company, Sparks, USA) in Petri dishes and were re-plated in 2-week cycles. Conidia were harvested from 10- to 14-day-old cultures approximately 12 hours before trials commenced and stored at 4°C. The surface of a culture was washed with sterile deionised water amended with Tween 20 (Sigma-Aldrich, St Louis, Missouri, USA) at a concentration of 0.01 mL.L^{-1}. Spore suspensions were adjusted with a spectrophotometer (Cecil 1011, Cecil Instruments Limited, Peterborough, UK) to an absorbance of 0.1 at 420 nm, which correlates with a

concentration of 1×10^6 spores.ml^{-1} (Morris and Nicholls, 1978; Eckert and Brown, 1986). The conidial suspensions were placed on magnetic stirrers to maintain a uniform suspension of spores during inoculation.

2.2. IMZ sensitivity of the nine PD and five PI isolates

The effective concentration that inhibits 50% mycelial growth (EC_{50}) of the various isolates was determined *in vitro*. Potato dextrose agar was amended with IMZ (Imazacure, 750 g.kg^{-1} SG, ICA International Chemicals, Stellenbosch, South Africa), which ranged in specific concentrations levels. Isolates were pre-screened for sensitivity and depending on the results, a specific range was used for each isolate. The ranges were 0, 0.005, 0.01, 0.015, 0.02, 0.03, 0.05, 0.1, 0.5 and 1 µg.mL^{-1} or 0, 1, 1.5, 2, 2.5, 3, 4 and 5 µg.mL^{-1}. Spore suspensions with a concentration of 1×10^6 spores.mL^{-1} were prepared for each isolate. A droplet of 20 µL spore suspension was placed in the middle of each specific amended PDA plate. The plates were incubated at 22°C for 5 days after which the colony diameters were measured twice perpendicularly. The percentage inhibition was calculated relative to colony diameters on unamended control plates for each specific isolate. Five replicate plates were prepared for each concentration per isolate and the trial was repeated three times. Percentage inhibition data of each isolate was regressed against IMZ concentration using non-linear regression with the function, Y = C/(1+Exp(-A-B*X)). The coefficient of determination (R^2) was used to demonstrate goodness of fit. EC_{50} value was calculated from the model for each isolate. This was done for each trial. The EC_{50} data from the three trials were subjected to analyses of variance and significant differences between the EC_{50} values of different isolates were compared by means of Fisher's test at a 95% confidence interval. All analyses were done using XLSTAT® (Addinsoft, www.xlstat.com). The resistance (R) factor for resistance isolates was determined by dividing the EC_{50} value of a resistant isolate by the mean EC_{50} value of the sensitive PD. From these results the isolates were arbitrarily categorised in terms of IMZ sensitivity as sensitive, low resistant, moderate resistant, resistant and highly resistant.

2.3. Effective IMZ residue levels for curative and protective control

2.3.1. Fruit

Untreated export quality Valencia sweet orange ('McClean' and 'Valencia Late') and navel orange ('Palmer' and 'Washington') fruit were obtained from various citrus packhouses in the Mpumalanga and Limpopo provinces of South Africa during the seasons of 2011 and 2012. Fruit were washed with an aqueous solution of 125 µg.mL^{-1} didecyl dimethyl ammonium chloride (Sprorekill, ICA International Chemicals, Stellenbosch, South Africa) and left to dry before it was stored at 3.5 – 7°C for ±3 days. A day before a trial, fruit were transferred from cold storage to ambient in order for fruit temperature to reach ambient and to allow any condensation to evaporate.

2.3.2. Imazalil treatment

Fruit were treated with an IMZ sulphate formulation (Imazacure, 750 g.kg^{-1} SG). A pilot trial was conducted first to determine the various IMZ concentrations needed to load a range of residue levels from very low (< 0.1 µg.g^{-1}) to very high (> 10 µg.g^{-1}) on the fruit following a 60-s dip at 22°C. From the pilot trial IMZ

concentrations of 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 µg.mL^{-1} were selected as the range that was used for treatments. The trial was repeated twice with all the PD isolates (PD1 – PD9) and three times with the PI isolates (PI1 – PI4 and PI7) on Valencia orange fruit. For comparative purposes, two isolates of PD (PD5 and PD9) were included with the PI isolates. Two similar trials were conducted on navel oranges with four PD isolates (PD3, PD5, PD6 and PD7) and two PI isolates (PI2 and PI4). Within each trial three replicate groups consisting of 12 fruit (six for curative control and six for protective control) were dipped at a time. Six extra fruit were added to two replicates for each treatment concentration per trial for IMZ residue analyses.

2.3.3. Residue analyses

Within 48 hours after each trial the fruit destined for residue analyses were prepared. For each replicate treatment, 6 fruit were cut into 4 equally sized pieces of which 3 were discarded and the rest weighed and macerated to a fine pulp by using a blender (Salton Elite Blender, Almalgamated Appliance Holdings Limited, Reuven, South Africa). The samples were frozen until analysis. Imazalil (chloramizol) residue analyses were conducted by Hearshaw and Kinnes Analytical Laboratory (Cape Town, South Africa). The samples were extracted using acetonitrile followed by a matrix solid phase dispersion extraction. The extracts were analysed using liquid chromatography tandem mass spectrometry (LCMS/MS; Agilent 6410, Agilent Technologies Inc., Santa Clara, California, USA). Residue values analysed from the different trials were subjected to an analyses of variance and Fisher's test at a 95% confidence interval using XLSTAT®.

2.3.4. Inoculation, incubation and evaluation

Fruit were treated protectively and curatively. Those destined for curative treatments were inoculated with the various isolates 24 h before treatment and those destined for protective treatment were first dip-treated, left to dry and then inoculated within ± 4 h after treatment. Fruit were simultaneously wounded and inoculated by dipping a stainless steel rod with a narrow, concave tip (2 mm long; 1mm diameter) into the spore suspension and then wounding the fruit rind through the flavedo into the top layer of the albedo. Four inoculated wounds were induced equal distances apart surrounding the calyx. After treatment and inoculation the fruit were incubated at 22°C. The fruit were incubated in lock back table grape cartons (APL cartons, Worcester, South Africa) on count SFT13 nectarine trays (Huhtamaki South Africa (Pty) Ltd, Atlantis, South Africa) and enclosed in transparent polyethylene bags.

Fruit were rated for infection and sporulation 5 and 10 days after inoculation, respectively. The number of infected wounds per fruit were evaluated using an ultra-violet light source (UV-A at 365 nm, Labino Mid-light; www.labino.com). Infected wounds could be identified as yellow fluorescence under UV light (Erasmus et al., 2011 / Chapter 2). Infection data were normalised to percentage control in relation to the untreated control. Sporulation was evaluated for each fruit as described by Erasmus *et* al. (2011; Chapter 2). Infected fruit were given a rating from 1 to 6 relating to the fruit area covered with green sporulation. Where 1 = infection with no sporulation, 2 = sporulation area less than 100 mm^2, 3 = sporulation area less than 50% of the fruit and more than 100 mm^2, 4 = sporulation area more than 50% of fruit and less than 75%, 5 = sporulation area more than 75% of the fruit and less 100%; and 6 = 100% covered with sporulating green mould. Infected fruit rated ≥ 4 were regarded as sporulating. Sporulation incidence per replicate (%) was calculated.

2.3.5. Calculating the effective residues for curative and protective control

Percentage control data for each replicate were regressed against residue levels of each specific trial using the non-linear function, Y = C/ (1+Exp (-A-B*X)), in XLSTAT®. The coefficient of determination (R^2) was used to demonstrate goodness of fit. The effective residue levels for 50% curative or protective control ($ER_{50}C$ and $ER_{50}P$, respectively) were calculated from the model for each isolate. The ER_{50} levels determined for each isolate in each trial were subjected to analysis of variance and the differences compared by means of Fisher's test at a 95% confidence interval. Data were analysed separately for the PD isolates on Valencia orange fruit, the PI isolates on Valencia orange fruit and for the selected PD and PI isolates on Navel orange fruit.

2.4. Efficacy of alternative fungicides against IMZ resistant isolates of P. digitatum

Three PD isolates, an IMZ sensitive, resistant and highly resistant (as determined in 2.2), were used and inoculated as described in 2.3.4. Fruit were treated curatively and protectively with 9 fungicides: SOPP (SOPP Super 20%, Advantage Agri Products, Paarl, South Africa), TBZ (ICA TBZ, ICA International Chemicals), GZT (Citricure, ICA International Chemicals), IMZ (Imazacure SG, ICA International Chemicals), PYR (Protector, ICA International Chemicals), FLU (Scholar, Syngenta, Midrand, South Africa), AZO (Ortiva, Syngenta), PLB (Janssen PMP, Belgium), GRA (Syngenta) and PPZ (Tilt, Syngenta) were applied in at concentrations registered in South Africa or at experimental concentrations, as 60 s dip treatments (Table 8). Fruit were incubated and evaluated as described in 2.1.3. Trials consisted of three replicates with six fruit and each trial was repeated three times each on navel and Valencia oranges. Percentage control data were subjected to analysis of variance using XLSTAT® and Fisher's test to compare differences at a 95% confidence interval.

3. Results

3.1. IMZ sensitivity of nine PD and five PI isolates

Non-linear regression with the function Y = C/ (1+Exp (-A-B*X)) resulted in very good fits for all isolates; R^2 values of > 0.84 (Table 1). There were significant differences between EC_{50} values of the various isolates ($P < 0.0001$; ANOVA not shown). PD5 had the highest level of resistance with an EC_{50} of 2.29 $\mu g.mL^{-1}$. PD1, PD4 and PD7 all had similar EC_{50} values (1.63, 1.87 and 1.62 $\mu g.mL^{-1}$, respectively; Table 1), but significantly lower than PD5. PD8 and PD2 had significantly lower EC_{50} levels than PD1, PD4 and PD7 and also differed significantly from each other (1.08 and 0.62 $\mu g.mL^{-1}$, respectively). PD3, PD6, PD9 and all the PI isolates had similar and significantly lower EC_{50} levels compared to the resistant isolates which ranged from 0.05 to 0.01 $\mu g.mL^{-1}$. Based on the Fisher's LSD test on EC_{50} values and their R factors the isolates were characterised as sensitive (PD3, PD6, PD9 and all the PI isolates), low resistant (PD2), moderately resistant (PD8), resistant (PD1, PD4 and PD7) and highly resistant (PD5).

3.2. Effective IMZ residue levels for curative and protective control

3.2.1. Valencia orange fruit - Penicillium digitatum

3.2.1.1. IMZ residue levels

Statistically similar residues were loaded following the 5 – 640 µg.mL^{-1} treatments (0.18 – 1.26 µg.g^{-1}; results not shown), and significantly higher residue levels loaded following dips in 1280 µg.mL^{-1} (5.43 µg.g^{-1}), and 2560 treatment (10.60 µg.g^{-1}).

3.2.1.2. ER_{50} levels

The R^2 values for the function, Y = C/ (1+Exp (-A-B*X)), were > 0.75 indicating very good fits (Table 2). Analysis of variance for the IMZ ER_{50}C and ER_{50}P values for the nine isolates showed a meaningful action × isolate interaction (*P* = 0.069; ANOVA not shown). The three sensitive isolates clustered together having the lowest ER_{50}C and ER_{50}P values (ranging from 0.26 – 0.29 µg.g^{-1} and 0.13 – 0.27 µg.g^{-1}, respectively; Table 2). For curative control, the low resistant, moderately resistant, one resistant (PD1) and the highly resistant isolates had similar ER_{50}C values (ranging from 1.22 – 1.91 µg.g^{-1}); these values were 4- to 7-fold higher than those for the sensitive isolates but were not significantly different. The other two resistant isolates (PD4 and PD7) had significantly higher EC_{50}C values (4.37 and 4.56 µg.g^{-1}, respectively) than all the isolates, but similar to the lower level of the highly resistant isolate (1.91 µg.g^{-1}). Protectively, the low resistant isolate clustered with two resistant isolates (PD4 and PD7) having significantly higher ER_{50}P values (2.94 – 4.46 µg.g^{-1}) compared to the moderately resistant (PD8) and one of the resistant isolates (PD1) with values of 1.08 and 1.00 µg.g^{-1}, respectively. The highly resistant isolate (PD5) had the highest ER_{50}P value (6.62 µg.g^{-1}), but not significantly higher than PD2 and PD7.

3.2.1.3. Sporulation

As certain treatments had zero infected fruit, data for the various sensitive and resistant isolates were pooled for statistical analyses. Analysis of variance for percentage sporulating fruit showed a significant isolate x concentration interaction (*P* = 0.032; ANOVA not shown). For the sensitive isolates there was generally no significant difference in sporulation levels on infected fruit between the untreated fruit and the IMZ treated fruit regardless of residue level (Table 3). Similarly, there was generally no statistical difference for the resistant isolates between the untreated and IMZ treated fruit. Interestingly, on fruit loaded with 0.18 and 0.24 µg.g^{-1} significantly lower sporulation incidence (33.3 and 27.8%, respectively) was observed than on fruit loaded with 5.43 and 10.60 µg.g^{-1} (80.6 and 79.6, respectively). A lower sporulation incidence was also evident for sensitive isolates on 0.18 µg.g^{-1} loaded fruit. A meaningful effect for curative or protective action was also observed (*P* = 0.112). The curative treatments had generally higher levels (> 15% overall) of sporulating fruit compared to the protective treatments.

3.2.2. Valencia orange fruit - Penicillium italicum *and two comparative PD isolates*

3.2.2.1. Residue levels

IMZ residue levels loaded following dips in concentrations of 5 – 320 µg.mL^{-1} increased from 0.07 – 0.61 µg.g^{-1} (results not shown). The highest three dip concentrations loaded significantly higher residue levels and differed significantly from each other (1.15, 2.31 and 5.53 µg.g^{-1} for 640, 1280 and 2560 µg.mL^{-1}, respectively).

3.2.2.2. ER_{50} levels

Data fitted the function, Y = C/ (1+Exp (-A-B*X)), relatively well (R^2 > 0.68; Table 4). Analysis of variance for the IMZ ER_{50} values showed a significant action × isolate interaction (P = 0.0001; ANOVA not shown). The ER_{50}P level for the highly resistant isolate, PD5 (4.38 µg.g^{-1}; Table 4), was significantly higher than its ER_{50}C level (1.47 µg.g^{-1}). Both these values were significantly higher than the rest of the sensitive PD and PI isolates' ER_{50}P and ER_{50}C levels (0.09 - 0.20 µg.g^{-1} and 0.11 – 0.26 µg.g^{-1}, respectively).

3.2.3. Navel orange fruit - Penicillium digitatum and P. italicum

3.2.3.1. Residue levels

IMZ residue levels loaded on navel orange fruit increased with treatment concentration: means of 0.13, 0.19, 0.22, 0.36, 0.56, 0.87, 1.37, 1.72, 3.09 and 5.64 µg.g^{-1} were loaded for the 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 µg.mL^{-1} treatments, respectively.

3.2.3.2. ER_{50} levels

The ER_{50}P values for PD5 and PD7 could not be determined, due to low levels of control (≈4%; result not shown). Therefore, ER_{50} data were analysed separately for curative and protective action. The R^2 values were > 0.74 for lines that were fitted by means of the function, Y = C/ (1+Exp (-A-B*X)) (Table 5 and 6). Analysis of variance for the ER_{50}C levels for green and blue mould caused by the 4 PD and 2 PI isolates indicated significant differences between isolates (P = 0.001; ANOVA not shown). The highly resistant and resistant isolate, PD5 and PD7, had significantly higher ER_{50}C levels (1.42 and 1.65 µg.g^{-1}, respectively) than the sensitive PD and PI isolates (0.21 – 0.37 µg.g^{-1}; Table 5); ER_{50}C values for the sensitive PD and PI isolates did not differ significantly. Analyses of variance for the ER_{50}P levels showed no significant difference (P = 0.213; ANOVA's not shown) between the ER_{50}P levels for IMZ sensitive PD and PI isolates, which were 0.28 - 0.35 and 0.15 – 0.29 µg.g^{-1}, respectively (Table 6).

3.2.3.3. Sporulation inhibition

Similar to section 1.1.1. data for the sensitive PD, resistant PD and sensitive PI isolates were pooled for statistical analyses. Analyses of variance for percentage sporulation incidence data showed a significant isolate x concentration interaction (P < 0.0001; ANOVA not shown). For sensitive PD isolates, there was no significant difference in sporulation incidence between the untreated fruit and IMZ treated fruit with residues of 0.13 – 0.56 µg.g^{-1}, where these levels ranged from 94.4 - 100.0% (Table 7). Treatments that loaded higher residue levels

(0.87 – 5.64 µg.g^{-1}) had significantly lower levels of sporulating fruit (76.9 – 26.4%). For the resistant PD isolates, no significant differences were found regardless of residue level and the sporulation incidence was > 90.0%. The sensitive PI isolates infected fruit showed a similar trend to the sensitive PD infected fruit, and significant reduction in sporulation incidence was observed from 0.19 – 1.72 µg.g^{-1} (63.7 – 16.9%) compared with the untreated control (95.8%). The ANOVA showed a significant isolate x action (curative and protective) interaction (P = 0.006; ANOVA not shown). Sporulation incidence on infected fruit levels did not differ between the curative and protective treatments when inoculated with resistant PD isolates (> 95.0%; results not shown), but curative and protective treatments of the sensitive PD isolates showed significantly lower sporulation levels (85.6 and 74.6%, respectively) and differed significantly from each other. The sensitive PI isolates showed significantly lower sporulation incidence for curative and protective treatments (46.9 and 42.3%, respectively).

3.3. Efficacy of alternative fungicides against IMZ resistant isolates of P. digitatum

3.3.1. Green mould control

Analysis of variance for percentage green mould control data showed a significant four factor interaction for citrus kind (navel and Valencia) × action (curative and protective) × isolate [sensitive (PD3), resistant (PD4) and highly resistant (PD5)] × fungicide (AZO, GRA, FLU, GZT, PLB, IMZ, PPZ, PYR, SOPP and TBZ) (P = 0.0002; ANOVA not shown). This difference could be mostly ascribed to Valencia trials having lower control levels compared to the navel trials. To simplify the interpretation the significant action x isolate x fungicide interaction was discussed (P < 0.0001) Curatively, SOPP, TBZ, GZT and PLB gave similar control levels to IMZ (> 90.0%; Table 8) of the sensitive isolate. PYR gave weaker control (78.0%) than these fungicides, but significantly higher than FLU, AZO, GRA and PPZ (< 39.0%). IMZ exhibited poor control of the two resistant isolates (35.3 and 30.3% for the resistant and highly resistant isolate, respectively). PPZ, TBZ and GZT treatment also resulted in very low control levels of these two isolates (< 39.0%). SOPP and PYR controlled the resistant isolates the best (> 90%). PLB gave ≈ 81.0% control of the resistant isolates, which was markedly lower than PYR and SOPP control of the resistant isolates, as well as PLB control of the sensitive isolate. The rest of the fungicides showed low control levels of < 48.0%.

Protectively, IMZ gave slightly poorer control (88.2%), but not significant poorer than its curative control of the IMZ sensitive isolate. GZT gave similar protective control (81.6%), while PLB gave the best protective control at 97.5%. SOPP gave much weaker protective control (19.6%) than curative, which could be ascribed to the rinsing of fruit immediately after SOPP treatment. TBZ and PYR gave significantly lower protective control (46.0 and 46.4%, respectively), compared to its curative treatments. FLU, AZO, GRA and PPZ gave slightly better protective control levels than curative, but even though it was significant in some cases the control levels were relatively low (<54.0%). IMZ, PPZ, TBZ and GZT failed to protectively control the IMZ resistant isolates (< 18.1%), while PLB also gave relative poor protective control (33.3 – 41.9%). GRA, PYR and AZO showed some protective control potential against the IMZ resistant isolates, but at varying levels (45.1 - 73.6%). Interestingly, control of the IMZ resistant isolates by these fungicides was markedly to significantly better than their control of the sensitive isolate (30.8 - 53.9%).

3.3.2. Sporulation

Sporulation data of one navel and three Valencia trials were combined. Analyses of variance for percentage sporulation incidence of infected orange fruit showed a significant action x fungicide and isolate x fungicide interaction were shown (P = 0.015 and < 0.0001, respectively; ANOVA not shown). However, the three factor isolate x action x fungicide interaction (P = 0.571) results are shown in Table 9. For the sensitive isolate, the levels of sporulation incidence on infected fruit were generally high (> 80.0), regardless of curative or protective treatment with most fungicides. However, IMZ treatment resulted in significant lower levels (62.8 and 69.4%, for curative and protective treatments, respectively), as well as curative and protective PLB treatment (23.6 and 2.8%, respectively). IMZ and PLB did not inhibit sporulation of the resistant isolates (>74.3%) and low levels of sporulation incidence were recorded only for SOPP (33.3%), but only in the curative treatments. For the rest of the fungicides the majority of levels were > 72.9 %, regardless of curative or protective treatment.

4. Discussion

Fungicide resistance monitoring and characterisation is generally conducted by means of *in vitro* growth studies (Staub and Sozzi, 1984; Russell, 2004). From these studies the baseline sensitivity for the given fungicide can be determined that forms the basis from where resistance development can be followed. In many citrus production regions including California (Holmes and Eckert, 1999), Florida (Brown, 1989), Uruguay (Pérez et al., 2011) and Morocco (Boubaker et al., 2009) the baseline sensitivity for IMZ has been established. All these studied determined the baseline sensitivity of IMZ to be < 0.05 $\mu g.g^{-1}$. To our knowledge no IMZ baseline has been determined for South Africa. *In* vitro fungicide sensitivity characterisation is rarely corroborated with *in vivo* characterisation (Wild, 1994; Kinay et al., 2007). Peréz et al. (2011) showed that isolates that would grow on 1 $\mu g.mL^{-1}$ IMZ *in vitro* would be able to overcome an IMZ residue of 3 $\mu g.g^{-1}$ loaded on citrus fruit; this was an attempt to establish the *in vitro* concentration by which practical resistance can be detected. This study is a first attempt to quantitatively categorise different PD and PI isolates based on *in vitro* as well as *in vivo* studies. It is clearly shown that the practical impact of a specific resistant isolate may differ significantly from what could be predicted or expected from *in vitro* categorisation.

Only one of the sensitive PD isolates (PD3) originated from a secluded orchard that has never been exposed to any postharvest fungicides. More isolates that have never been exposed to imazalil are required to establish a proper IMZ baseline sensitivity level (Russell, 2004; Kinay et al., 2007). Nonetheless, the average EC_{50} value of the sensitive PD isolates in this study (0.03 $\mu g.g^{-1}$) relates well to baselines determined in other work (Kinay et al., 2007; Holmes and Eckert, 1999; Brown, 1989; Pérez et al., 2011).

Hamamoto et al. (2000) suggested that the *PdCYP51* gene can be expressed in increasing levels that will render higher levels of DMI resistance. Ghosoph et al. (2007) confirmed these findings and alluded to the same conclusion that the level of *PdCYP51* expression could be related to the level of IMZ resistance. So far three *CYP51* genes that contributes to IMZ resistance have been characterised: IMZ-R1 (Hamamoto et al., 2000), IMZ-R2 (Ghosoph et al., 2007) and IMZ-R3 (Sun et al., 2013). Through molecular characterisation, all the resistant isolates in this study were characterised as the IMZ-R3 genotype (Mareli Kellerman, pers. comm.).

From the EC_{50} values it was shown that the IMZ resistant isolates in our study had different levels of IMZ resistance with R-factors of 19 to 71. Theoretically, an isolate with an R-factor of >2 can be considered resistant (Delp and Dekker, 1985). R-factors typically vary from as low as 5 to 100 (Brent and Hollomon, 2007). R-factor values obtained in our study may be considered to be small when compared to those found with other fungicides, where the differences in terms of sensitivity levels are much wider (Staub, 1991). Even though the difference between the sensitive and resistant PD isolates in this study could be considered relatively low, the effect on disease control on fruit was quite substantial. Furthermore, the ER_{50} values, which were determined *in vivo* on fruit, did not fall into these distinct categories as determined *in vitro*.

The sensitive PD isolates had similar ER_{50} values regardless of action (curative of protective) treatment or citrus type (< 0.37 $\mu g.g^{-1}$). However, the $ER_{50}C$ and $ER_{50}P$ values of the resistant isolates could not be predicted from their EC_{50} values. On Valencia, two resistant isolates, PD4 and PD7, showed the highest level of resistance in curative treatment ($ER_{50}C$ levels of 4.37- 4.56 $\mu g.g^{-1}$), while the highly resistant isolate, PD5, had a significantly lower $ER_{50}C$ level of 1.91 $\mu g.g^{-1}$, which was similar to the low and moderately resistant isolates. For protective control, however, this highly resistant isolate, as well as the low resistant isolate had the highest $ER_{50}P$ values. The low resistant and highly resistant isolates behaved similarly on Valencia with lower $ER_{50}C$ (1.65 and 1.91 $\mu g.g^{-1}$, respectively) and higher $ER_{50}P$ values (4.46 and 6.62 $\mu g.g^{-1}$, respectively). The other resistant isolates had similar respective values for curative and protective ER_{50} values.

In vitro EC_{50} values did not correlate with *in vivo* ER_{50} values, and this was further complicated when different ER_{50} values were obtained from different citrus types. Infection levels of the resistant isolates on navel oranges were so severe that $ER_{50}P$ values could not be determined. The highly resistant isolate behaved similarly in curative treatments on navel and Valencia ($ER_{50}C$ values of 1.91 and 1.42 $\mu g.g^{-1}$, respectively), but the resistant isolate had lower ER_{50} values on navel compared with Valencia (1.65 and 4.56 $\mu g.g^{-1}$, respectively). In cases with high ER_{50} values, it could be assumed that a residue level of > 5 $\mu g.g^{-1}$ (exceeding the IMZ MRL) would be required to completely control the resistant isolates. Also the isolate that were categorised as low resistant proved to be "highly resistant" with an $ER_{50}P$ value of 4.46 $\mu g.g^{-1}$. If isolates with this level of resistance prevail in an environment where fruit are stored in a long term protocol and repacked; failure of control can be expected (Holmes and Eckert, 1999).

In vitro categorisation of fungal populations is commonly used as a diagnostic measure of fungicide resistance. For PD, a discriminatory concentration of 1.0 $\mu g.mL^{-1}$ IMZ amended PDA was suggested for commercial resistance monitoring (Pérez et al., 2011). Our work indicates that this level could be reduced to 0.5 $\mu g.mL^{-1}$. While none of the sensitive isolates in our study was able to grow at 0.5 $\mu g.mL^{-1}$, the low resistant PD isolate had an EC_{50} of 0.62 $\mu g.mL^{-1}$ and EC_{95} of 1.05 $\mu g.mL^{-1}$ (results not shown). As this isolate will be substantially inhibited at 1.0 $\mu g.mL^{-1}$, it might be regarded as sensitive in a discriminatory *in vitro* assay. Importantly, this isolate had ER_{50} values of 1.65 and 4.46 $\mu g.g^{-1}$ (for curative and protective treatment, respectively), which will cause loss of control in a packhouse as demonstrated in this study.

At the onset of the study, a collection of sensitive and resistant PI isolates was sourced for inclusion in the study. However, following *in vitro* and *in vivo* characterisation, all the PI isolates proved to be sensitive. Imazalil resistant isolates of PI are usually less prevalent than *P. digitatum* (Eckert, 1990; Bus et al., 1991; Holmes and Eckert, 1999). One reason for this could be due to the fact that PD grows much faster than PI at

temperatures 15°C - 28°C (Eckert and Eaks, 1989). These temperatures are predominant during harvest time in most citrus regions. It was shown that resistant PD isolates were not less virulent compared to sensitive PD isolates, but less competitive (Holmes and Eckert, 1995).

Residue levels varied between the different trials. Imazalil residue levels on Valencia orange fruit were approximately double in the trial where all nine PD isolates were used compared to the trial where the PI isolates were involved (0.24 -10.6 $\mu g.g^{-1}$ compared to 0.07 – 5.53 $\mu g.g^{-1}$ in dip treatments that ranged from 5 – 2560 $\mu g.mL^{-1}$ for the respective trials). The IMZ residue levels on navel oranges were similar to those on the second Valencia orange trial mentioned above (0.13 – 5.64 $\mu g.g^{-1}$). It is suspected that the fluctuation in residue loading can be ascribed to fluctuating municipal water quality, where an increase in the pH level of water can have an increasing effect on residue loading in an IMZ sulphate solution (Erasmus et al., 2011, 2013 / Chapter 2 & 3). Regrettably, the pH levels of IMZ sulphate solutions used in this study were not monitored. Despite variation in residue loading between fruit types and batches, the sensitive and highly resistant isolates (PD9 and PD5, respectively) were used as reference isolates and relatively comparable ER_{50} values were determined in the different trials.

This project was not specifically designed to study sporulation or its inhibition as was done in previous studies (Brown et al., 1983; Brown and Dezman, 1990); however, trends in sporulation inhibition were observed between treatments. On Valencia, no differences were found between curative and protective treatments, but there was a tendency for the resistant PD isolates to have lower sporulation incidences on infected fruit with lower residue levels (27.8 – 67.6% on 0.18 – 0.89 $\mu g.g^{-1}$), compared to the sensitive PD isolates (all > 66.2%). In contrast, sensitive PD infections showed lower levels of sporulation on navel fruit with higher residue levels (26.4 – 68.1% on 1.37 – 5.64 $\mu g.g^{-1}$), while resistant PD isolates had levels of > 90% sporulation regardless of residue level. The sensitive PI isolates had the lowest level of sporulation; on fruit with residues of 1.37 and 1.72 $\mu g.g^{-1}$, sporulation incidences were 12.0 and 16.9%, respectively. This apparently enhanced sporulation inhibition effect by IMZ could in part explain why resistant PI isolates are less prevalent than those of PD. The poor inhibition of sporulation on fruit infected with resistant isolates, regardless of residue level is problematic and shows that increasing the IMZ residue level in order to combat resistance is not an effective practice. This was also shown in previous studies where a residue of ≈ 5 $\mu g.g^{-1}$ could not lead to sporulation inhibition of a resistant isolate (Erasmus et al., 2011, 2013 / Chapter 2 & 3; Eckert, 1990).

The conventional green mould fungicides (IMZ, SOPP, TBZ, GZT, PYR and PLB) gave excellent curative control (≥ 90.0%) against sensitive PD isolates. PYR and SOPP equalled this level of curative control of the resistant isolates. PLB also performed well with > 80% curative control of the resistant isolates. Protectively, only PLB, IMZ and GZT stood out giving levels of > 80.0% control of the sensitive isolate infections, while none of the fungicides were as effective against the resistant isolate infections. The highest protective control levels on these isolates resulted from PYR and GRA (≈ 60 and ≈ 70%, respectively). The protective action of green mould fungicide can possibly be improved by an alternative application method. This was the case for IMZ, where applicationin wax coatings resulted in better protective control compared to dip treatments (Njombolwana et al., 2013a, 2013b).

Multiple resistance was observed in two IMZ resistant isolates (PD4 and PD5). GZT, TBZ and PPZ were ineffective in controlling the IMZ resistant isolates, while providing significantly better control of the

sensitive isolate. These fungicides have been in use longer than IMZ and resistance in the PD population is well known. Resistance to GZT and TBZ was reported in 1983 in Australia (WILD, 1983). To our knowledge, this is the first report of multiple resistance to IMZ, GZT, PPZ and TBZ in PD. Multiple resistance have been reported for IMZ, TBZ and SOPP (Holmes and Eckert, 1999). Imazalil and PPZ fall in the same DMI class, and cross resistance has been shown between these two fungicides (Mckay et al., 2012). Some indication of potential negative cross resistance was also observed where the IMZ resistant isolates were markedly better controlled with GRA, PYR and AZO than their respective control of the sensitive isolates. This will, however, have to be investigated further.

The poor control of the IMZ sensitive isolate with PPZ in our study could be due to the 24 h incubation period being too long. Good results were obtained on infection of up to16 h (McKay et al., 2012).

The older fungicide SOPP proved to be a very good alternative for curative control, but cannot be considered for protective control as fruit needs to be rinsed after treatment to prevent phytotoxicity. If the pH is controlled the risk of phytotoxicity may be reduced. The pH should be managed at a level of 12, and lowering the pH will increase residue loading (Dezman et al., 1986). Protective control by SOPP in our trials was relatively poor, but the pH was not adjusted to 12.

The excellent curative control by PYR reported in this work confirm other work showing this fungicide as a favourable IMZ alternative (Adaskaveg et al., 2005; D'Aquino et al., 2006; Smilanick et al., 2006). Unfortunately, relatively poor protective control was achieved.

PLB provided excellent curative and protective control of IMZ sensitive isolates and relatively good curative control of IMZ resistant isolates. However, the significantly weaker control of IMZ resistant isolates, especially protectively, was most probably due to IMZ resistance, as well as the lower PYR concentration in at the formulated product (500 $\mu g.mL^{-1}$), which is 50% lower that PYR is recommended as stand-alone product. Moreover, Lado et al. (2010) recommended 750 $\mu g.mL^{-1}$ PLB as an effective concentration, while 500 $\mu g.mL^{-1}$ (the registered concentration) was evaluated in this study. Schirra et al. (2010) got very good results with a combination of 600 $\mu g.g^{-1}$ each of IMZ and PYR.

AZO, FLU and GRA gave relatively poor curative control (< 62%). Kanetis et al. (2007) showed that these actives showed very good potential on infections of 21 h and younger. In our work the infections was 24 h old, which is realistically comparable to industry situations where fruit can stand for longer than a day after harvest before the first fungicide application. D'Aquino et al. (2013) showed excellent curative control (≤ 15% infection) with 600 $\mu g.mL^{-1}$ FLU on 24 h old green mould infections. The differences between our work and that of D'Aquino et al. (2013) might be ascribed to different inoculation methods, as they induced 2 mm wide by 2 mm deep wounds before dipping the fruit in a spore suspension of 1×10^5 $spores.mL^{-1}$. In our study, fruit were wound-inoculated simultaneously by dipping the wound-inducer in a suspension of 1×10^6 $spores.mL^{-1}$ prior to wounding; this resulted in approximately 4×10^4 spores deposited at each wound site, which might be more severe than found in the field. Schirra et al. (2010) used a similar inoculation method to D'Aquino et al. (2013), but their spore suspension was 1×10^6 $spores.mL^{-1}$. They could only achieve ≈ 12 – 13% control with 30 and 60 s dip treatments in 600 $\mu g.g^{-1}$ FLU and AZO. Other fungicides, however, were able to control infections that originate from this type of inoculation. Protectively these actives showed some potential, especially GRA giving 73.6 and 63.1% control of infections from the two respective resistant isolates in this study. Interestingly,

protective control of the sensitive isolate was markedly to significantly poorer than that of the resistant isolates following treatment with GRA, FLU, AZO and PYR. One of the attributes of FLU and AZO is the inhibition of conidium germination (; Bushong and Timmer, 2000; Rosslenbroich and Stuebler, 2000 Kanetis et al., 2007), which might explain the protective ability of these fungicides.

The sporulation inhibition effect of IMZ is better expressed when it is applied in wax coatings than when applied in an aqueous solution (Erasmus et al., 2011, 2013 / Chapter 2 & 3; Njombolwana et al., 2013a, 2013b). Brown and Dezman (1990) showed that fruit with an intact wax layer and treated with IMZ had lower levels of sporulation compared to fruit with the wax layer removed. Residues levels of > 2 $\mu g.g^{-1}$ are required for the inhibition of sporulation where the IMZ EC formulation was used (Brown and Dezman, 1990; Smilanick et al., 1997). So far no consistent trends could be observed in terms of sporulation control with aqueous IMZ sulphate treatments. PYR has also been shown to have the ability to inhibit or reduce green mould sporulation, but not as well as IMZ (Smilanick et al., 2006). In their study, the combination of IMZ and PYR showed sporulation inhibition of an IMZ sensitive isolate of *P. digitatum* applied within wax or aqueous solution. Our work confirms this as PLB was able to control sporulation the best on the sensitive isolate infections (23.6 and 2.8% sporulation for curative and protective, respectively). This might be ascribed to a synergistic effect between IMZ and PYR, as sporulation inhibition for these actives individually was relatively poor. However, sporulation inhibition by PLB was relatively poor for the resistant isolate infections, and comparable to PYR alone. Kanetis et al. (2007) also found that PLB was unable to inhibit sporulation on green mould caused by IMZ resistant isolates. The only fungicide that showed some level of sporulation inhibition of the resistant isolates was SOPP, but for curative treatment only (33.3%).

In Chapter 4, it has been shown that post dip-treatment brushing can reduce > 90% of the potential residue to levels of < 0.5 $\mu g.g^{-1}$. This level gave good curative control of the sensitive isolate (PD3), but poor inhibition of sporulation due to the reduced residue levels. The protective ability of the reduced residue loads was also questioned. In this study, the $ER_{50}C$ and $ER_{50}P$ values for sensitive PD isolates from 0.20 - 0.33 $\mu g.g^{-1}$ confirms the good curative control at low residue levels, but also indicate that good protective control of IMZ sensitive isolates could also be achieved. In the absence of IMZ resistance, green mould can therefore be effectively controlled if fruit is dip-treated within 24 h after harvest with relatively low residue levels. However, it can be anticipated that older infections will escape control (Chapter 4) and their sporulation will not be inhibited.

This work shows the importance of loading an effective IMZ residue to combat the green and blue mould. Although sensitive isolates could be controlled with < 0.5 $\mu g.g^{-1}$, sporulation inhibition was mostly observed at higher residue loads (> 2 $\mu g.g^{-1}$). The ideal IMZ residue level of 2 – 3 $\mu g.g^{-1}$ was, however, not effective against all resistant isolates, but should reduce infection and inoculum buildup in packhouse environments. Our work showed that further increasing IMZ residue levels will not be effective in improving control of resistance PD isolates, especially considering the MRL restriction. In packhouses where IMZ resistance is prevalent, fungicides with alternative modes of action could be applied; although none of these fungicides equalled IMZ in its combined curative, protective and sporulation control abilities of IMZ sensitive PD isolates, they would be effective when applied in an integrated programme. Each fungicide's optimal application needs to be specifically determined, as the profound effect of application on residue loading and control has

been demonstrated in previous studies (Erasmus et al., 2011, 2013 / Chapter 2 & 3; Njombolwana et al., 2013a, 2013b).

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Table 1. Effective imazalil (IMZ) concentration that inhibits 50% mycelia growth (EC_{50}) and resistance factors (R factor) of nine *Penicillium digitatum* (PD) and five *P. italicum* (PI) isolates that were determined following mycelium growth measurements on potato dextrose agar amended with IMZ at concentrations ranging from 0 to 5 µg.mL^{-1} from which percentage growth inhibition data for each isolate and IMZ concentrations were subjected to non-linear regression using the function, Y = C/ (1+Exp (-A-B*X).

Sensitivity category[a]	Function variables and standard deviations[b]				EC_{50} (µg.mL^{-1})[c]	R factor[d]
and isolate	A	B	C	R^2		
Sensitive						
PI4	-4.5 (2.0)	1132.7 (602.4)	84.5 (5.2)	> 0.85	0.005 e	
PI1	-4.8 (2.5)	1167.9 (801.2)	93.2 (1.6)	> 0.95	0.005 e	
PI7	-6.7 (4.4)	3338.9 (4124.6)	87.1 (2.8)	> 0.90	0.007 e	
PI2	-4.0 (1.8)	174.1 (124.2)	98.0 (1.3)	> 0.88	0.027 e	
PD6	-23.7 (13.9)	991.7 (643.3)	100.9 (1.2)	> 0.99	0.027 e	
PD3	-5.1 (1.1)	135.1 (28.9)	87.6 (36.6)	> 0.98	0.032 e	
PD9	-7.8 (4.5)	192.2 (74.7)	101.4 (1.5)	> 0.84	0.038 e	
PI3	-4.6 (1.6)	91.2 (30.6)	100.8 (1.0)	> 0.99	0.050 e	
Low resistant						
PD2	-4.2 (1.0)	6.7 (1.5)	101.3 (1.4)	> 0.97	0.615 d	19.0
Moderately resistant						
PD8	-17.6 (10.2)	17.4 (10.5)	100.5 (0.7)	> 0.98	1.075 c	33.2
Resistant						
PD7	-7.6 (0.7)	4.7 (0.1)	99.9 (0.1)	> 0.95	1.618 b	50.0
PD1	-15.4 (1.3)	9.5 (1.2)	100.0 (0.2)	> 0.99	1.634 b	50.5
PD4	-6.6 (1.2)	3.5 (0.5)	101.4 (0.2)	> 0.90	1.865 b	57.6
Highly resistant						
PD5	-26.2 (25.2)	9.5 (7.0)	100.8 (0.6)	> 0.99	2.290 a	70.7

[a]The resistant isolates were categorized using the R factor and the Fisher's LSD test.

[b]Mean values of three trials, where lines were fitted on the data from 5 replicates per trial.

[c] Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

[d]The R factor was determined by dividing the EC_{50} value of a resistant isolate by the mean EC_{50} value of the three sensitive PD isolates (PD3, PD6 and PD9).

Table 2. Effective imazalil (IMZ) residue values for predicted 50% curative ($ER_{50}C$) and protective ($ER_{50}P$) control of green mould caused by nine *Penicilium digitatum* (PD) isolates respectively inoculated 24 h prior to or ± 4 h after treatment on Valencia orange fruit dipped in a range of IMZ concentrations (0 – 2560 µg.mL^{-1}) at 22°C for 60 s. The ER_{50} values were calculated from the function, Y = C/ (1+Exp (-A-B*X)), of which the parameters were determined from regression of percentage control data for each isolate on IMZ residue derived from the inoculation trials.

Isolate[a]	Function variables and standard deviances[b]			R^2	ER_{50} (µg.g^{-1})[c]
	A	B	C		
Curative					
Sensitive					
PD3	-4.8 (0.9)	19.4 (1.5)	88.5 (1.7)	> 0.92	0.26 ab
PD6	-3.0 (0.8)	11.5 (4.6)	94.8 (0.8)	> 0.89	0.29 ab
PD9	-4.5 (0.2)	11.8 (8.4)	100.6 (10.9)	> 0.90	0.26 ab
Low resistant					
PD2	-4.4 (2.3)	4.8 (3.8)	88.8 (3.5)	> 0.81	1.65 abc
Moderately resistant					
PD8	-4.7 (0.7)	9.4 (4.3)	94.3 (3.5)	> 0.92	1.22 ab
Resistant					
PD1	-4.0 (0.7)	3.7 (2.0)	99.6 (0.4)	> 0.94	1.40 abc
PD4	-2.5 (0.1)	0.7 (0.2)	85.3 (14.8)	> 0.82	4.37 de
PD7	-2.5 (0.2)	0.8 (0.4)	84.7 (7.0)	> 0.79	4.56 de
Highly resistant					
PD5	-5.5 (2.5)	2.2 (0.7)	1252.9 (1173.9)	> 0.87	1.91 abcd
Protective					
Sensitive					
PD3	-3.7 (1.6)	19.8 (4.7)	93.7 (2.4)	> 0.84	0.19 a
PD6	-3.4 (0.5)	14.2 (3.4)	95.2 (6.4)	> 0.91	0.27 ab
PD9	-7.7 (4.2)	91.7 (71.6)	92.3 (3.6)	> 0.82	0.13 a
Low resistant					
PD2	-2.2 (0.2)	0.7 (0.4)	85.9 (0.2)	> 0.75	4.46 de
Moderately resistant					
PD8	-4.7 (0.5)	6.0 (1.6)	65.8 (8.9)	> 0.81	1.08 ab
Resistant					
PD1	-8.0 (0.9)	8.1 (1.4)	98.8 (0.0)	> 0.95	1.00 ab
PD4	-5.5 (2.9)	2.2 (0.9)	69.6 (2.5)	> 0.90	2.94 bcd
PD7	-2.7 (0.3)	0.8 (0.3)	85.0 (0.9)	> 0.84	4.00 cde
Highly resistant					
PD5	18.4 (20.8)	-6.5 (7.0)	52.3 (47.6)	> 0.79	6.62 e

[a]Each isolate was categorized in terms of IMZ sensitivity as indicated by their EC_{50} values.

[b]Mean values of two trials, where lines were fitted on the data from three replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 3. Mean sporulation incidence (%) on infected Valencia orange fruit that were inoculated with three imazalil (IMZ) sensitive and six IMZ resistant *Pencillium digitatum* isolates, and curatively and protectively dip-treated in IMZ solutions ranging from 0 to 2560 µg.mL^{-1} to effect a range of IMZ residues on fruit.

IMZ residue (µg.g^{-1})	Sporulation incidence on infected fruit (%)[a]	
	Sensitive isolates	Resistant isolates
0.00	83.1 abc	70.8 bcde
0.18	66.2 cde	33.3 de
0.20	92.5 ab	63.8 cde
0.24	88.1 abc	27.8 e
0.30	85.2 abc	57.4 cde
0.57	100.0 a	62.3 cde
0.89	98.6 a	67.6 cde
1.20	89.6 abc	72.3 bcd
1.26	80.0 abc	71.5 bcde
5.43		80.6 abc
10.60		79.6 abc

[a] Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 4. Effective imazalil (IMZ) residue values for predicted 50% curative ($ER_{50}C$) and protective ($ER_{50}P$) control of green mould caused by two *Penicilium digitatum* (PD) and five *P. italicum* (PI) isolates respectively inoculated 24 h prior to or ± 4 h after treatment on Valencia orange fruit dipped in a range of IMZ concentrations (0 – 2560 µg.mL^{-1}) at 22°C for 60 s. The ER_{50} values were calculated from the function, Y = C/(1+Exp(-A-B*X)), of which the parameters were determined from regression of percentage control data for each isolate on IMZ residue derived from the inoculation trials.

Isolate[a]	Function variables and standard deviances[b]			R^2	ER_{50} (µg.g^{-1})[c]
	A	B	C		
Curative					
Sensitive					
PD9	-2.6 (1.2)	22.3 (15.1)	92.7 (3.5)	> 0.88	0.15 a
PI1	-2.0 (0.2)	12.3 (2.4)	96.4 (2.1)	> 0.83	0.18 a
PI2	-2.8 (0.4)	11.3 (1.0)	93.2 (1.6)	> 0.87	0.26 a
PI3	-3.1 (0.9)	23.9 (9.9)	95.8 (2.0)	> 0.81	0.14 a
PI4	-3.0 (1.8)	31.2 (19.3)	95.5 (3.8)	> 0.76	0.11 a
PI7	-13.5 (15.8)	137.1 (176.7)	89.6 (1.7)	> 0.77	0.17 a
Highly resistant					
PD5	-4.0 (0.6)	5.2 (3.4)	75.8 (9.5)	> 0.78	1.47 b
Protective					
Sensitive					
PD9	-3.0 (1.4)	41.0 (26.3)	93.0 (2.0)	> 0.81	0.11 a
PI1	-3.0 (0.7)	31.5 (19.8)	93.1 (0.6)	> 0.84	0.12 a
PI2	-3.1 (0.6)	17.1 (3.2)	88.0 (4.6)	> 0.88	0.20 a
PI3	-2.2 (0.4)	13.8 (3.9)	97.3 (1.8)	> 0.90	0.17 a
PI4	-3.4 (0.7)	36.2 (8.2)	95.8 (1.1)	> 0.69	0.10 a
PI7	-7.3 (4.3)	86.7 (57.0)	96.1 (1.3)	> 0.85	0.09 a
Highly resistant					
PD5	-4.2 (1.3)	1.4 (0.2)	47.6 (14.0)	> 0.68	4.38 c

[a]Each isolate was categorized in terms of IMZ sensitivity as indicated by their EC_{50} values.

[b]Mean values of three trials, where lines were fitted on the data from three replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 5. Effective imazalil (IMZ) residue values for predicted 50% curative ($ER_{50}C$) control of green mould caused by four *Penicilium digitatum* (PD) and two *P. italicum* (PI) isolates respectively inoculated 24 h prior to treatment on navel orange fruit dipped in a range of IMZ concentrations (0 – 2560 µg.mL^{-1}) at 22°C for 60 s. The ER_{50} values were calculated from the function, Y = C/ (1+Exp (-A-B*X)), of which the parameters were determined from regression of percentage control data for each isolate on IMZ residue derived from the inoculation trials.

Sensitivity category[a]	Isolate	Function variables and standard deviances[b]			R^2	$ER_{50}C$ (µg.g^{-1})[c]
		A	B	C		
Sensitive	PD3	-3.9 (0.7)	12.3 (3.3)	94.2 (3.8)	> 0.89	0.37 a
	PD6	-2.2 (0.7)	9.3 (4.8)	98.7 (0.5)	> 0.85	0.28 a
	PI2	-3.5 (0.9)	12.0 (3.9)	91.3 (4.2)	> 0.93	0.37 a
	PI4	-17.6 (15.3)	106.1 (97.5)	97.5 (0.0)	> 0.94	0.21 a
Resistant	PD7	-25.9 (22.2)	15.3 (12.5)	68.1 (12.6)	> 0.93	1.65 b
Highly resistant	PD5	-5.1 (0.4)	4.0 (0.6)	78.6 (3.5)	> 0.88	1.42 b

[a]Each isolate was categorized in terms of IMZ sensitivity as indicated by their EC_{50} values.

[b]Mean values of two trials, where lines were fitted on the data from three replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 6. Effective imazalil (IMZ) residue values for predicted 50% protective ($ER_{50}P$) control of green mould caused by two *Penicilium digitatum* (PD) and two *P. italicum* (PI) isolates respectively inoculated ± 4 h after treatment on navel orange fruit dipped in a range of IMZ concentrations (0 – 2560 $\mu g.mL^{-1}$) at 22°C for 60 s. The ER_{50} values were calculated from the function, Y = C/ (1+Exp (-A-B*X)), of which the parameters were determined from regression of percentage control data for each isolate on IMZ residue derived from the inoculation trials.

Sensitivity category[a]	Isolate	Function variables and standard deviances[b]			R^2	$ER_{50}P$ ($\mu g.g^{-1}$)[c]
		A	B	C		
Sensitive	PD3	-3.1 (1.0)	12.3 (5.5)	96.3 (2.1)	> 0.90	0.28 a
	PD6	-2.3 (0.2)	6.7 (0.4)	96.9 (0.4)	> 0.91	0.35 a
	PI2	-7.7 (5.3)	23.5 (10.3)	94.1 (2.6)	> 0.74	0.29 a
	PI4	-5.8 (2.2)	42.2 (19.0)	96.3 (1.2)	> 0.85	0.15 a

[a]Each isolate was categorized in terms of IMZ sensitivity as indicated by their EC_{50} values.

[b]Mean values of two trials, where lines were fitted on the data from three replicates per trial.

[c]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 7. Mean sporulation incidence (%) on infected Navel orange fruit that were inoculated with 2 imazalil (IMZ) sensitive *Penicillium digitatum* (PD), two IMZ resistant PD and two IMZ sensitive *P. italicum* (PI) isolates, and curatively and protectively dip-treated in IMZ solutions ranging from 0 to 2560 $\mu g.mL^{-1}$ to affect a range of IMZ residues on fruit.

IMZ residue ($\mu g.g^{-1}$)	Sporulation incidence on infected fruit (%)[a]		
	Sensitive PD isolates	Resistant PD isolates	Sensitive PI isolates
0.00	100.0 a	100.0 a	95.8 ab
0.13	100.0 a	99.3 a	89.5 ab
0.19	99.3 a	100.0 a	63.7 c
0.22	99.3 a	100.0 a	60.3 cd
0.36	95.0 ab	94.4 ab	42.7 de
0.56	94.4 ab	91.7 ab	36.7 ef
0.87	76.9 bc	99.3 a	24.0 efg
1.37	68.1 c	90.1 ab	12.0 g
1.72	57.4 cd	98.3 a	16.9 fg
3.09	26.4 efg	96.9 a	
5.64	64.4 c	95.4 ab	

[a] Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 8. Mean percentage green mould control on orange fruit inoculated with an imazalil (IMZ) sensitive, resistant or highly resistant *Pencillium digitatum* isolate 24 h prior to (curative) or ± 4 h after (protective) 60 s aqueous dip treatment with 10 different fungicides at registered or experimental concentrations.

Action	Product	Concentration ($\mu g.g^{-1}$)	Green mould control (%)[a]		
			IMZ sensitive isolate	IMZ resistant isolate	IMZ highly resistant isolate
Curative	Imazalil (IMZ)	500	92.1 abc	35.3 lmnop	30.3 opq
	Sodium o-phenylphenate	20000	97.9 a	98.2 a	99.1 a
	Thiabendazole	1000	92.5 ab	3.1 wxy	2.3 xy
	Guazatine	1000	90.0 abc	38.6 klmno	24.0 pqrs
	Pyrimethanil	1000	78.0 de	90.4 abc	93.9 a
	Philabuster®	500	93.8 a	81.0 cde	81.2 bcde
	Fludioxonil	500	12.2 tuvwxy	29.5 opqr	18.5 rstu
	Azoxystrobin	500	8.9 uvwxy	19.4 qrstu	12.4 tuvwxy
	Graduate®A^{+}	500	30.7 nopq	47.5 ijk	37.7 klmno
	Propiconazole	500	38.9 klmno	14.2 stuvw	10.0 tuvwxy
Protective	Imazalil	500	88.2 abcd	1.7 xy	1.1 y
	Sodium o-phenylphenate	20000	19.6 qrstu	13.0 stuvwx	15.9 stuv
	Thiabendazole	1000	46.0 ijkl	3.0 wxy	3.2 wxy
	Guazatine	1000	81.6 bcde	18.1 rstu	5.1 vwxy
	Pyrimethanil	1000	46.4 ijkl	59.7 gh	57.2 ghi
	Philabuster®	500	97.5 a	41.9 klmn	33.3 nop
	Fludioxonil	500	15.0 stuv	34.2 mnop	20.5 qrst
	Azoxystrobin	500	30.8 nopq	55.9 ghij	45.1 jklm
	Graduate®A^{+}	500	53.9 ghij	73.6 ef	63.1 fg
	Propiconazole	500	48.8 hijk	1.6 xy	3.2 wxy

[a] Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)

Table 9. Mean sporulation incidence (%) on infected orange fruit inoculated with an imazalil (IMZ) sensitive, resistant or highly resistant *Pencillium digitatum* isolate 24 h prior to (curative) or ± 4 h after (protective) 60 s aqueous dip treatment with 10 different fungicides at registered or experimental concentrations.

Action	Product	Concentration ($\mu g.mL^{-1}$)	Sporulation incidence oninfected fruit (%)[a]		
			IMZ sensitve isolate	IMZ resistant isolate	IMZ highly resistant isolate
Curative	Imazalil	500	62.8 def	98.6 l	100.0 l
	Sodium o-phenylphenate	20000	100.0 l	33.3 cd	33.3 abc
	Thiabendazole	1000	80.7 fghijkl	98.6 l	100.0 l
	Guazatine	1000	83.3 fghijkl	91.4 ghijkl	100.0 l
	Pyrimethanil	1000	84.7 fghijkl	53.9 cde	72.9 defghi
	Philabuster®	500	23.6 ab	74.3 defghij	93.3 hijkl
	Fludioxonil	500	100.0 l	88.9 ghijkl	100.0 l
	Azoxystrobin	500	100.0 l	84.4 fghijkl	100.0 l
	Graduate®A+	500	84.0 fghijkl	71.5 defgh	98.6 l
	Propiconazole	500	94.4 hijkl	98.6 l	100.0 l
	Water control		100.0 l	100.0 l	100.0 l
	Untreated control		100.0 l	100.0 l	100.0 l
Protective	Imazalil	500	69.4 defg	100.0 l	100.0 l
	Sodium o-phenylphenate	20000	93.7 hijkl	79.2 fghijkl	89.4 ghijkl
	Thiabendazole	1000	93.3 hijkl	100.0 l	100.0 l
	Guazatine	1000	92.8 ghijkl	98.3 kl	100.0 l
	Pyrimethanil	1000	98.6 l	85.7 fghijkl	96.9 jkl
	Philabuster®	500	2.8 a	95.8 ijkl	100.0 l
	Fludioxonil	500	87.5 ghijkl	74.7 efghijk	100.0 l
	Azoxystrobin	500	100.0 l	79.2 fghijkl	95.3 hijkl
	Graduate®A+	500	89.4 ghijkl	50.6 cd	92.1 ghijkl
	Propiconazole	500	95.8 ijkl	98.3 kl	100.0 l
	Water control		100.0 l	100.0 l	100.0 l
	Untreated control		100.0 l	100.0 l	100.0 l

[a]Each value followed by the same letter is not significantly different according to Fisher's LSD test ($P \leq 0.05$)